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**WORKSHOP ON ALTERNATIVES TO ANIMALS IN RESEARCH**  
**(DEFENCE RESEARCH ESTABLISHMENT SUFFIELD)**

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**October 1989**

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*Handwritten:* Key to Toxicology (KP)

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FOREWORD BY DR. G.H. KIMBELL

CHIEF, DEFENCE RESEARCH ESTABLISHMENT SUFFIELD

On behalf of Dr. Schofield, the Chief of Research and Development for DND, I would like to welcome you to this Workshop on Alternatives to Animals in Research. In particular, I would like to welcome the keynote speakers, to thank them for donating their time and to acknowledge the financial support of the Canadian Council on Animal Care. I note that the meeting is well attended by representatives from other government departments and the local universities. Finally, I am pleased to see that many of our own DRES staff are here to learn and to participate. DRES is pleased to provide a forum for this Workshop and expects to reap the benefits for years to come. I am acutely aware of the ethical and economic reasons why DRES should attempt to reduce its present dependence on animals as research subjects. Once again, I bid you all welcome and wish you success in your deliberations.

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WORKSHOP ON ALTERNATIVES TO  
ANIMALS IN RESEARCH

DEFENCE RESEARCH ESTABLISHMENT  
SUFFIELD, RALSTON, ALBERTA  
16 - 17 SEPTEMBER 1987

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PROGRAM SCHEDULE

Wednesday, September 16, 1987

- 0830 Welcome by Dr. George Kimbell, Chief DRES
- 0835 "Application of *in vitro* Tests to R & D in Pharmaceutical and  
Pharmaceutical and Chemical Industries  
- presented by Dr. D. Ilse
- 0910 "Tissue Cultures"  
- presented by Dr. S. Fedoroff
- 0945 Coffee Break
- 1015 "Application of Tissue Cultures to Biomedical Research"  
- presented by Dr. S. Fedoroff
- 1050 "The Use of Animal Alternatives in the Safety Evaluation Process"  
- presented by Dr. G.L. Plaa
- 1145 Lunch
- 1315 "Animals or replacements, a CCAC Perspective"  
- presented by Dr. H.C. Rowsell
- 1405 Break
- 1415 Roundtable Discussion - The Chairman will call upon each of the  
four speakers in turn to answer questions. Before each question  
period he will briefly summarize the speaker's presentation.
- 1530 Adjourn
- 1900 Reception and Dinner at the Travelodge (No Host)

Thursday, September 17, 1987

- 0900 Round Table Discussion - This discussion will be more general,  
but will attempt to provide conclusions and recommendations for  
an official report of the workshop.
- 1200 Lunch
- 1300 Tour of DRES and meetings with individual scientists (to be  
arranged)
- 1600 Visitors Depart

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INVITED SPEAKERS

Dr. S. Fedoroff  
Professor and Head  
Department of Anatomy, College of Medicine  
University of Saskatchewan  
Saskatoon, Saskatchewan

Dr. D. Ilse  
Director of Pharmaceutical Research  
Ortho Pharmaceuticals (Canada) (Ltd.)  
Don Mills, Ontario

Dr. G.L. Plaa  
Professor  
Department of Pharmacology  
University of Montreal  
Montreal, Quebec

Dr. H.C. Rowsell  
Executive Director  
Canadian Council on Animal Care  
Ottawa, Ontario

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PREFACE

The DRES Animal Care Committee (DRES/ACC) conceived the idea for a workshop to examine alternatives to live animals in a research setting as a natural course of the deliberations involved in approving the applications to use experimental animals. While the committee recognizes the necessity of the use of live animals, there is also a desire to ensure that animals are being used in the most economical fashion and that unavoidable distress or pain to the animals is minimized. These discussions arose in part due to the nature of the research programme at DRES and in part because it is a component of our mandate to encourage research using alternative methods.

The DRES/ACC invited recognized experts in the field of alternatives to animals in research who would share with DRES scientists and staff the innovative approaches currently in use and those envisaged for the future, the options to existing animal intensive tests such as the LD<sub>50</sub> determination and, most importantly, convince the scientists of the suitability and reliability of the alternatives available. Drs. Rowsell, Federoff, Plaa and Ilse certainly achieved these goals in their formal presentations and in their stimulating and thought-provoking answers during the discussion periods and the subsequent roundtable. We are particularly pleased to learn that our workshop at DRES has stimulated other institutions to present similar seminars on animal alternatives and use.

The DRES/ACC would like to express its appreciation to the Canadian Council on Animal Care for its support of this workshop, to Dr. G. Kimbell, Chief/DRES and Dr. W.S. Myles, Director/Defence Sciences Division, for their support and suggestions and, of course, to the speakers for their expert advice and suggestions. Finally, our

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thanks to the scientists and staff at DRES and the guests that attended the seminar who, through their thoughtful questions and lively participation in the discussions guaranteed the success of the workshop.

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THE VALUE OF SHORT-TERM TESTS IN REDUCING THE USE OF CHEMICALS  
IN THE COURSE OF NEW DRUG DEVELOPMENT

Derek Ilse  
Ortho Pharmaceutical (Canada) Ltd.

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Derek Ilse, D.Sc.

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Dr. Ilse is a member of various scientific societies and has served as an Assistant Professor of Pharmacology (University of Manitoba), a senior biochemist (South African Institute for Medical Research) and a lecturer (University of New South Wales, Sydney, Australia). He has served as a postdoctoral research fellow in biochemistry (School of Medical Research, St. Vincent's Hospital, Melbourne, Australia) and research officer (South African Council for Scientific and Industrial Research). To date, he has published 15 scientific papers.

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THE VALUE OF SHORT-TERM TESTS IN REDUCING THE USE OF CHEMICALS  
IN THE COURSE OF NEW DRUG DEVELOPMENT

Derek Ilse

Ortho Pharmaceutical (Canada) Ltd.

In recent times, many concerns have been expressed regarding the use of animals for routine testing of products, or as experimental models in research. Critics unfamiliar with the complexities of Research and Development (R&D), or with the legal obligations associated with pharmaceutical manufacturing sometimes believe that animal experiments are either unnecessary, or are badly planned, offering little justification for the pain and suffering inflicted, or the animal lives expended. Today, I will not be saying much about academic research, but will present some information on the use of experimental animals in the pharmaceutical industry and will try to show how much really is done to minimize the use of animals or to optimize the circumstances and conditions under which they are used. I will also mention some of the efforts which are being made to find alternatives to the use of experimental animals.

In the pharmaceutical industry, the nature and the course of work is affected not only by market demands or competitive pressures within the industry, but also to a large degree, by government consumer-protection legislation (Fig. 1). Under these circumstances, laboratory animals are used not only for the discovery and development of new drugs, but also for the assurance of safety and quality of the finished, licensed product, whether it be a drug or a medical device (Fig. 2). The development of a new drug is a complex process, which nowadays may take from eight to twelve years and may cost as much as

\$100 million. The most critical steps in the development process are shown in Fig. 3, whereas Fig. 4 demonstrates the co-ordination of the various development phases within the time frame which may extend from discovery, to the time of a first submission of R&D information to regulatory agencies such as the Health Protection Bureau (HPB) of the Canadian Government's Department of Health and Welfare, or the U.S. Food and Drug Administration (FDA). The extent and the quality of drug SAFETY TESTING is rigorously controlled by HPB and FDA guidelines, as well as by government regulations for Good laboratory Practices (GLP) in many countries. Failure to comply with these regulations would usually bar a new drug from the final review process which is needed to support its licensing and eventual introduction to the market. Many of the safety tests mentioned in Figs. 3 and 4 involve the use of experimental animals and are mandatory under the above-mentioned regulations since at the present time, there are no validated, non-animal procedures which would be suitable for evaluation of the pharmacological effects of the drug on a system as complex as the mammalian body. This does not mean that the industry is always satisfied with these tests. On the contrary, we find that they often take a very long time and are consequently very expensive (Fig. 5). Furthermore, there frequently is disagreement on the significance of results which emerge from such studies.

There have been many suggestions that *in vitro* procedures should be used as an alternative to animal experimentation (Fig. 6). In several instances, such procedures have actually proved highly practical, especially in the initial screening of newly-synthesized compounds for a specific pharmacological activity, or in the examination of specific aspects of the mechanisms of action of a particular drug. This stage of the drug development process is not so rigorously regulated by legislation, so that diagnostic efficacy, cost-effectiveness, and short

duration have been of the most important factors responsible for the proliferation and increased use of *ex vivo* and *in vitro* procedures during the early stages of new drug development (Fig. 7).

In evaluating diagnostic efficacy and possible eventual usefulness of such short-term tests, scientists must answer a number of critical questions (Fig. 8). Some of these actually confront us with a serious dilemma: How could *ex vivo* tests on fresh, isolated animal tissues, or other *in vitro* procedures with microorganisms or with cultured, immortalized cell lines, possibly provide any realistic forecast of how a drug might affect the complete animal? If the newly-designed compound is intended to eliminate a virus or a pathogenic microorganism, or to change the performance of a discrete stage or sub-stage of an easily measurable biological mechanisms (Figs. 9-14), then an appropriate short-term test would be of great advantage in improving the success rate in the screening process. This is where they have been most successful as is evidenced by the rapid proliferation of useful, well-designed new drugs on the market in recent years. Such use of short-term tests has had a great impact on the number of animals used for drug discovery and development purposes. A good example is found in our own Canadian corporation: By now it is well-known, that even in the face of adverse patent legislation, we have increased our yearly research expenditure several fold over the past fifteen years to accommodate an increase and diversification of pharmaceutical research interests. Even so, our use of animals for screening and other research purposes has decreased so much through the use of *ex vivo* procedures with fresh cells and tissue extracts or by the *in vitro* application of immortalized cell lines (Fig. 15), that our overall animal usage in 1986 amounted to only one fifth of what it was in 1972 (Fig. 16). We may therefore conclude that when selectively used for screening purposes or for mechanistic studies, short-term tests will continue to reduce the use of animals in

simple, primary screening tests and will save a great deal of time and money for the developer of new drugs (Fig. 17).

In some phases of drug development, short-term tests are not very useful, especially in the developmental phases which involve a study of bio-availability pharmacokinetics, metabolism and general pharmacological and toxicological profiles. For establishing dose size and possible dosing frequency, one needs to use live animals and should monitor the metabolism and disposition of the drug in the animal to facilitate the proper planning of appropriate drug safety studies. Are short-term tests useful for the study of drug safety? At the present, the answer is "yes" and "no". In our own facility, as in most other progressive pharmaceutical corporations, a number of relatively simple *in vitro* tests have been used to obtain a first indication of possible toxicity (Fig. 18). The first five listed here, are useful for pre-screening of drugs so that any with significant toxicity may be put aside and not introduced into animal studies at all. Such selective use of these specialized procedures is therefore also contributing much to the reduced use of live animals in the early evaluation of drug safety. Unfortunately, these tests are not 100% reliable, since a cell which is cultured *in vitro*, is exposed to a drug perhaps at concentrations which would differ significantly from those which would prevail in the whole animal, where natural mechanisms of metabolic transformation and detoxication may contribute to the reduction of the actual toxicological hazard during the careful use of the drug. Nevertheless, it would be better to eliminate some drugs which are not really toxic, as false positives in such tests, than to find out much later on, after the use of many animals and the expenditure of very large sums of money, that they are toxic after all. On the other hand, what about compounds which show up "negative" in primary *in vitro* screening tests? We would not accept such a

result as final evidence for lack of toxicity nor would any of the regulatory agencies. The last three test categories mentioned in Fig. 18, have been submitted to much scrutiny in recent years in an international collaborative study program administered under the auspices of the World Health Organization (Ref. 1). The consensus seems to be that, useful as these tests may be for primary assessment of toxicity and elimination of strongly "positive" compounds, they are not sufficiently reliable to reassure drug developers, or the regulatory agencies such as the HPB or the FDA, of safety (Figs. 19 & 20). That is why the pharmaceutical industry cannot possibly use such *in vitro* procedure as replacement for the live-animal tests which currently are in use (Fig. 21). Some of the tests which may be usefully applied for early detection of potential mutagens and carcinogens, are listed in Fig. 22, together with the estimated reliability of those procedures (Ref. 2). The best known of these tests, i.e., the Ames Test, makes use of a variety of prokaryotic and eukaryotic strains of microorganisms and serves as a useful primary screen for potential mutagens, but users must nevertheless be aware of its shortcomings (Fig. 23 & Ref. 2). While the cell-transformation test is regarded as useful for *in vitro* assessment of potential carcinogenicity, it should be noted that theoretical understanding of the process of cell transformation and subsequent progression to a distinct neoplasm *in vivo*, is undergoing frequent revision as research progresses. Consequently, interpretation of results from *in vitro* cell transformation tests should proceed on the basis of the latest theoretical concepts. In conclusion, it may be said that in recent years, the use of live animals for screening and investigation of new drugs has been significantly reduced through the selective use of short-term tests. However, validation of most short-term tests is not yet at a stage which would support their unaided use for assessment of drug safety.

While they may usefully provide supporting information, favourable review of New Drug Submissions in Canada will for many years still have to rely on the satisfactory performance of the new drug when evaluated in the customary live-animal safety tests, according to well established guidelines.

#### REFERENCES

1. Summary report on the evaluation of short-term tests for carcinogens (collaborative study on *in vitro* tests), Environmental Health Criteria 47, World Health Organization, Geneva, 1985.
2. Lave, L.B. & Omenn, G.S.: Cost-effectiveness of short-term tests for carcinogenicity, Nature, 324 (1986), p. 29.

DEVELOPMENT OF PHARMACEUTICALS AND INDUSTRIAL CHEMICALS

IS REGULATED BY:

1. Food and Cosmetics Act
2. Safety of Industrial Chemicals Act
3. Health and Welfare Regulations
4. Environmental Protection Legislation
5. Market Demands
6. Competition

Figure 1

USE OF ANIMALS FOR INDUSTRIAL RESEARCH

1. New Drug Screening
2. Drug Safety Evaluation
3. Mechanistic and Drug Disposition Studies
4. Quality Control
5. Development of New Biomedical Devices

Figure 2

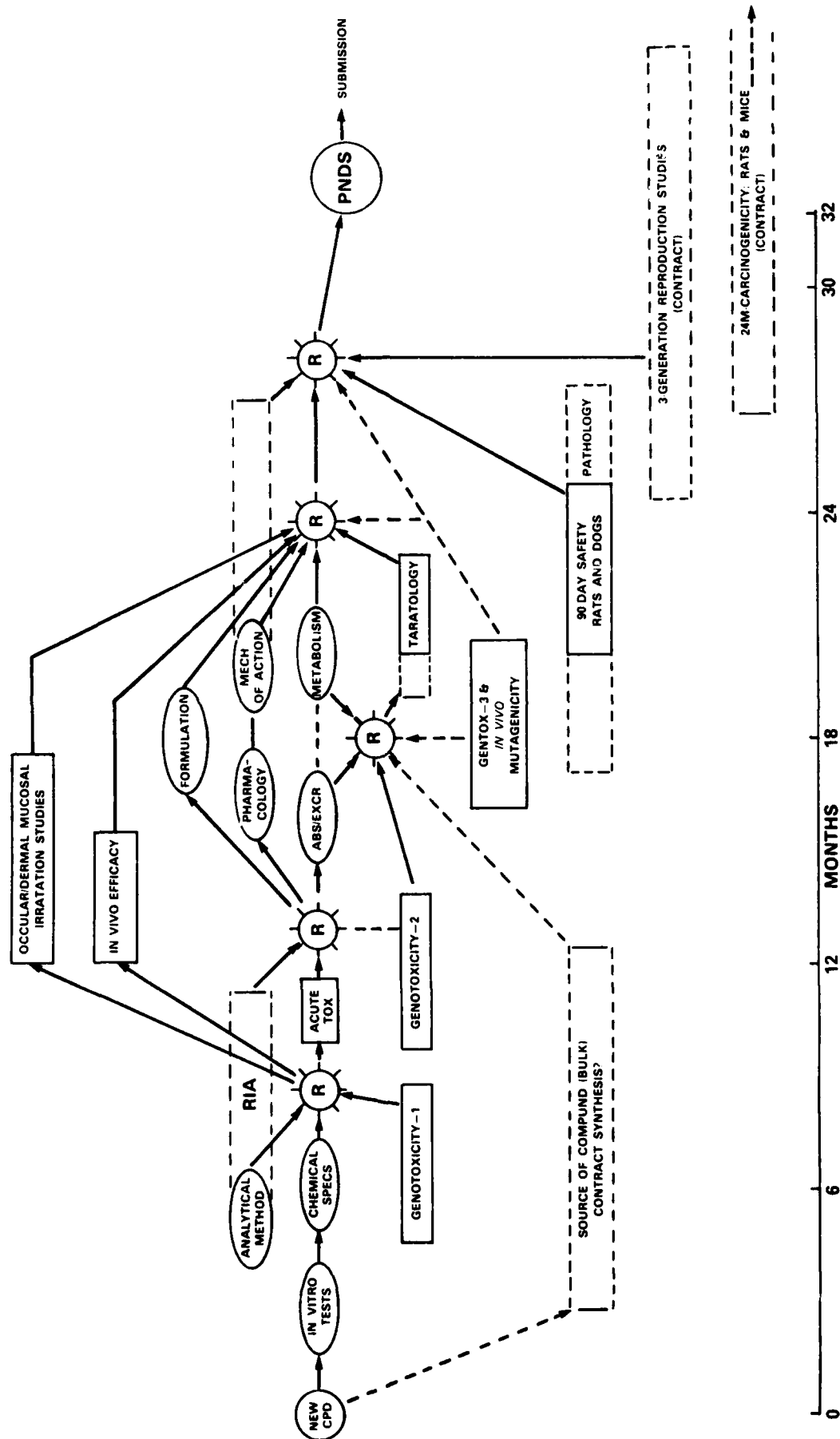
CRITICAL STEPS IN NEW DRUG DEVELOPMENT

- PHASE 1: Selective Synthesis
- PHASE 2: What Activity *in vitro*?  $ED_{50}$   
What toxicity *in vitro*?  $TD_{50}$   
Safety Index
- PHASE 3: Basic Mutagenicity Tests *in vitro*  
– Ames  
– Cell Transformation
- PHASE 4: Acute Toxicity *in vivo*  
"Two Mouse" Model  
Single Dog Model
- PHASE 5: Efficacy *in vivo*  
(appropriate model)
- PHASE 6: Absorption/Excretion Profile  
– best route for administration?  
– what formulation?  
– most appropriate dose?  
– what dosing regimen?
- PHASE 7: Subacute Toxicity  
Teratology  
Additional *in vitro* mutagenicity tests
- PHASE 8: Chronic Toxicity  
Multigeneration reproduction studies

VALIDATED  
ASSAY FOR  
DRUG IN BLOOD

Figure 3

## PRE-NDS STUDIES IN THE DEVELOPMENT OF A NEW DRUG



SOME FACTORS WHICH REDUCE DESIRABILITY OF ANIMAL STUDIES

FREQUENT LACK OF DIAGNOSTIC PRECISION

THEY ARE SLOW

THEY ARE EXPENSIVE

Figure 5

EVENTS WHICH HAVE LED TO DEVELOPMENT OF

IN VITRO PROCEDURES

1. Great advances in basic scientific knowledge: Demand for closer look at biological mechanisms.
2. Search for molecular events which control initiation and normal maintenance of physiological processes.
3. Animal testing is
  - slow
  - expensive
  - superficial
  - frequently indecisive
4. Need for acceleration in the production of drugs and industrial chemicals:
  - Demand for greater efficiency in testing.
5. GLP regulations.

Figure 6

*IN VITRO/EX VIVO*

(Hours-days-weeks)

"SHORT-TERM TESTS"

*IN VIVO*

(One month or less)

Figure 7

CRITICAL QUESTIONS REGARDING SHORT-TERM TESTS

1. How can short-term tests be used to predict and explain potential toxicological risks for humans and domestic animals?
2. What short-term tests are currently available? How much weight should be placed on each?
3. What is the current state of validation of each?
4. How well do short-term tests predict toxicity?
5. What degree of confidence do short-term tests add to the results of animal bioassays in the evaluation of toxicological risks?
6. Can a battery of tests be developed which would provide reasonable predictions of toxicity and, if so, what criteria should be met by such a battery?
7. How cost-effective would such tests be?

Figure 8

IN VITRO TESTS

Promotion of Cell Development

Antimicrobial

Antifungal

Antiviral

Figure 9

EX VIVO ASSAYS

Cellular Immunoassays  
Promotion of Cell Development  
Specific Receptor Assays  
    – competitive binding  
Specific Enzyme Assays  
Hepatocyte Culture Assay  
Skin Penetration Assays

Figure 10

EX VIVO IMMUNOASSAYS

Lymphocyte Transformation

Lymphocyte – Suppression  
– Activation

(Suppressor and Helper Cell Function)

Macrophage Migration Inhibition

Neutrophil Chemotaxis

Neutrophil Aggregation

Specific Antibody Synthesis

Figure 11

SPECIFIC ENZYME ASSAY

(IN VITRO AND EX VIVO)

Cyclic Nucleotide Phosphodiesterase

Angiotensin Converting Enzyme

Plasma Renin Activity

Phospholipase A<sub>2</sub>

Alkaline Phosphatase

Acid Phosphatase

Prostaglandin Synthetase

15 – Hydroxy Prostaglandin Dehydrogenase

Arachidonic Acid Metabolism

$\Delta^5$  – Lipogenase Assay

Na<sup>+</sup> K<sup>+</sup> – ATPase

Ornithine Decarboxylase

Figure 12

RECEPTOR ASSAYS

Androgen

Estrogen

Progestin

PGF<sub>2</sub> (Corpus Luteum)

SRS-A (Ileum)

Neuroleptic

Muscarinic Cholinergic

Dopamine

Benzodiazepine

$\alpha_1$ - &  $\alpha_2$ -Adrenergic

$\beta_1$ - &  $\beta_2$ -Adrenergic

5-HT<sub>1</sub> & 5-HT<sub>2</sub> (In Brain)

Figure 13

OTHER ASSAYS *IN VITRO*

*Inhibition of  $^{45}\text{Ca}$  Accumulation (Erythrocytes)*

Inhibition of Platelet Aggregation

Serotonin Release

Histamine Release

RIA for  $\text{TXB}_2$

6-Keto Prostaglandin  $\text{F}_1$

$^3\text{H}$ -Thymidine Uptake by Guinea Pig Skin

Drug Penetration through Guinea Pig Skin

Figure 14

CELL CULTURES USED

(Primary and Immortalized)

Vero

Hela

Rat Granulosa Cells

PRK

BHK

Lymphoblastoid

Parietal Cells

Osteoblasts

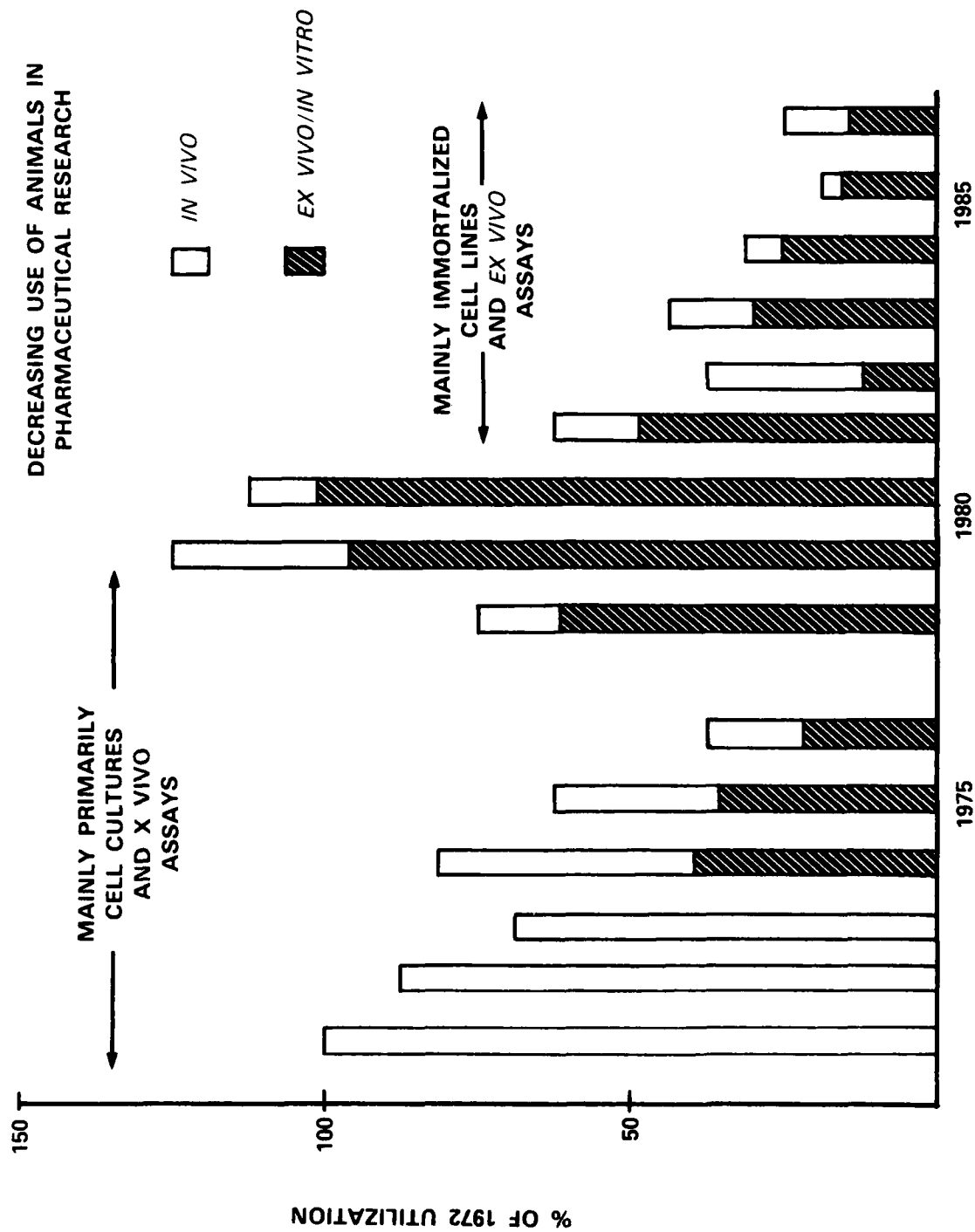
L 929

Lewis Lung Sarcoma

P 815

Bone Marrow Cells

Figure 15



ADVANTAGES INHERENT IN THE USE OF

*IN VITRO* PROCEDURES

1. They may represent a critical, course and rate-limiting step of a biological process.
2. They usually provide results quickly.
3. Can be performed in great numbers by relatively few technologists.
4. Cost-effectiveness.

Figure 17

IN VITRO TOXICITY TESTS

Morphological changes

Dye-exclusion tests

<sup>3</sup>H-Thymidine uptake

<sup>14</sup>C-Leucine Uptake

Sar-Probability Tests

Carcinogenicity

Teratogenicity

Mutagenicity

Figure 18

....TO ESTABLISH AN ASSAY AT THE LEVEL OF INTERNATIONAL  
ACCEPTANCE, REQUIRES ABOUT A DECADE OF METICULOUS  
SCIENTIFIC ENDEAVOUR AND INTERNATIONAL COLLABORATION

W.H.O., GENEVA 1985\*

\*"Summary Report on the evaluation of Short-term  
Tests for Carcinogens (Collaborative Study on  
*In Vitro* tests)"')

Figure 19

A BIOLOGICAL SYSTEM, WHICH CAN BE A  
POWERFUL AND FLEXIBLE TOOL IN THE  
HANDS OF AN EXPERIENCED RESEARCH  
WORKER, CANNOT BE EASILY TRANSFORMED  
INTO THE SOMEWHAT INFLEXIBLE  
PROCEDURE THAT IS REQUIRED FOR A  
TEST SYSTEM FOR ROUTINE USE  
THROUGHOUT THE WORLD.

W.H.O., GENEVA 1985

Figure 20

OBJECTIONS TO *IN VITRO* TESTS

1. Results are not always reconcilable with whole-body responses to the drug.
2. Regulatory agencies (FDA & HPB) frequently accept only results which cast doubt upon the safety or efficacy of the experimental compound, *e.g.*, negative results in even a large battery of *in vitro* tests are seldom accepted as unequivocal evidence for lack of drug-associated toxicity.

Figure 21

SUGGESTED PRIMARY TEST SET FOR PREDICTION OF  
CARCINOGENICITY/MUTAGENICITY

1. Salmonella/Microsome
2. Cell transformation
3. Unscheduled DNA Synthesis

Reliability: About 80%

Approx. Cost: \$18,700

Figure 22

Extensive, co-operative international study programs have not yet arrived at clear-cut conclusions concerning a single complementary eukaryotic assay that is capable of giving a positive response for carcinogens found negative in the standard salmonella assay.

Figure 23

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TISSUE CULTURE

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Sergey Fedoroff, Ph.D., D.Sc

Professor

Department of Anatomy

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Dr. Fedoroff is a member of numerous scientific societies and has served them in various capacities. He has been a member and appointed to various committees of the Medical Research Council of Canada, Canadian Council on Animal Care, Saskatchewan Health Research Board, W. Alton Jones Cell Science Center (Lake Placid, New York), Pasadena Foundation for Medical Research (Pasadena, California), World Health Organization, Canada Science Secretariat, American Type Culture Collection (Rockville, Maryland) University of Panama, and Institute of Developmental Neuroscience and Aging (Denver, Colorado). Dr. Fedoroff has also served as a member of the editorial boards on In Vitro, Journal of Tissue Culture, Future of Health Perspectives, International Journal of Developmental Neuroscience, Advances in Cellular Neurobiology, Cellular Neurobiology and Journal of Neurosciences. He has received numerous honors and awards, including the Federal Medical Faculty Award and Queen's Silver Jubilee Medal. He has presented and published over 100 scientific papers.

TISSUE CULTURE

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INTRODUCTION

The complexity of living multicellular organisms is so great that study of their components *in situ* is difficult. Biomedical research that developed in this century is to a great extent based on the principle of reductionism, i.e., separating the organism into its component parts: first into organs, then tissues, cells, cell organelles and finally into its component macromolecules.

Tissue culture is considered to have begun with Harrison's experiments on nerve fibers in 1907 and is limited, in the scale of reductionism, to tissues and cells. The cardinal rule in Tissue Culture, the Critical Cubic Millimeter, is the critical size of tissue that can be grown in cultures. Because there is no blood circulation in tissue cultures, nutrition is accomplished by diffusion of fluids through the tissue and fluids can successfully diffuse through tissue of not more than one cubic millimeter in size. If larger, necrosis develops in the center. The term "organ culture" is misleading because

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Presented at the Workshop on Alternatives, Suffield, Alberta, September 16-17, 1987; sponsored by the Canadian Council on Animal Care and the Defence Research Establishment, Suffield.

in reality, since size is the limiting factor, only tissues of an organ, not the whole organ, can be grown in culture. When a tissue fragment of one cubic millimeter or less is planted in culture, individual cells can grow out from the fragment making it possible to observe single cells under the highest powers of magnification, over a long period of time.

#### CELL INTERACTION AND MICROENVIRONMENT

Multiple interactions occur between various cell types in the living organism. The survival and function of cells depend on these interactions which may be classified as systemic interactions, mediated via hormones over a distance; homotypic interactions, which occur between similar cells over very short distances and for which close proximity of the cells is a prerequisite; and heterotypic interactions which are similar to homotypic ones, but take place between cells of different types. When a fragment of tissue or disaggregated cells are planted in cultures they become separated from the continuous supply of nutrients via blood and from systemic interactions. Therefore, a prerequisite for growing cells in cultures is provision of a microenvironment that, ideally, simulates the *in situ* environment. The following must be considered: substrata, metabolic substrata, pH, ionic strength, osmolality, gas phase (oxygen/CO<sub>2</sub>) and instructive messengers.

#### Substrata

When cells are planted in culture, they must attach to the substratum. It is now realized that the nature of the substratum is of the utmost importance for cell differentiation and function. Originally, Pyrex glass was used, but it has been replaced by plastic

specially prepared for tissue culture work. Recently, more and more natural substrates are being used, e.g., collagen, polylysine, polyornithine, amnion, allantois, basement membrane from cornea, vitreous humor, and in special situations, laminin, fibronectin or commercially prepared mixtures of components found in basement membranes, e.g. Basement Membrane Matrigel and Extracellular Matrix (ECM).

#### Metabolic Substrata

Many culture media formulated for specific cell types are available commercially. They vary in complexity but all contain inorganic salts, amino acids, vitamins, an energy source and a buffer. Some may also contain trace elements, lipids, purines and pyrimidines, hormones and growth factors. Some companies, e.g., Gibco Laboratories, provide catalogues listing various chemically defined media. It is advisable to consult the appropriate literature before selecting a medium.

#### pH

In most situations the pH of the culture medium is regulated by the use of bicarbonate buffer and a CO<sub>2</sub>-rich (5-10%) atmosphere. This requires the use of special incubators in which appropriate temperatures, humidity and CO<sub>2</sub> concentration are maintained. Cultures are grown in loosely stoppered petri dishes or flasks to facilitate air exchange.

Sodium  $\beta$ -glycerophosphate buffer does not depend on the CO<sub>2</sub> environment and therefore any type of incubator can be used and cells can be grown in tightly stoppered containers. Although rarely used, this buffer has many advantages.

### Ionic Strength and Osmolality

It is important that cells are exposed to fluids which are isotonic to the cells. The ionicity is not necessarily the same for every cell type. The normal range of osmolality for media is between 300-320 mosM/Kg. Osmolality may change during culturing and the degree of change depends on culturing procedures used.

### Gas Phase

Most commonly, 5-10% CO<sub>2</sub> in air is provided. The concentration of CO<sub>2</sub> depends on the concentration of bicarbonate in the medium and the pH desired. Certain cells, however, require a high concentration of oxygen.

### Instructive Messengers

All cells are interdependent and the degree of their differentiation and function depends on the instructive message they receive, either in the form of ligands which bind to cell receptors, or direct cell-to-cell interactions through gap junctions or extracellular matrix. This area is presently being intensively investigated. We still know relatively little about communication between cells.

### CULTURING PROCEDURES

Cells in cultures can be divided into two large groups: anchorage-dependent and anchorage-independent. Culturing procedures to be used depend on the group to which cells belong.

Anchorage-Dependent Cells

The following procedures are suitable for anchorage-dependent cells: Fragment cultures, cell cultures, clonal cultures, microcarrier cultures, hollow fiber cultures, encapsulated cell cultures, roller bottle cultures, petri dish cultures and flask cultures.

Fragment Cultures

Fragment cultures consist of small tissue explants, not larger than one cubic millimeter, in flasks or petri dishes or in special culture assemblies, e.g., Rose chambers. In such cultures, cells grow out of the explant and it is mainly the outgrowing cells that are studied. In special situations, cell differentiation within the explant is of interest. In such cases cell outgrowth is suppressed and the fragments must be embedded and cut into thin sections for study.

Cell Cultures

The tissues are disaggregated with enzymes (trypsin, collagenase, etc.) or by mechanical means (putting through nylon or steel mesh). The resulting cell suspensions, in appropriate dilutions, are planted in flasks or petri dishes. In most cases a monolayer of cells will form.

Colony Cultures

These cultures are prepared in a way similar to that for cell cultures, except that petri dishes are inoculated with smaller numbers of cells. Cells attach to the substratum, proliferate, and form discrete colonies.

The size of the inoculum is important to avoid overlapping of the colonies. This method may be used to assess plating efficiency of cells and as a quantitative bioassay.

#### Microcarrier Cultures

To increase the number of cells produced in cultures, the surface area of the substratum must also increase. This can be accomplished by putting into the flask beads with special surfaces. The cells then grow on the inner surface of the flask and also completely cover the beads, producing very large yields of cells.

#### Hollow Fiber Cultures

Specially prepared fibers are commercially available in plastic devices. Culture medium is perfused through the openings of the fibers and the cells grow on the surfaces of the fibers or within the walls of the fibers. These are three dimensional and tend to simulate *in situ* blood-tissue relationships.

#### Encapsulation Cell Cultures

It is possible to coat a cell or group of cells in a capsule made of alginate-polylysine-alginate or Enapcel<sup>R</sup> material. In these cultures interaction between cells is prevented; however, the products secreted by the cells and nutrients from the medium can penetrate the capsules.

Roller Bottle Cultures

Originally, roller tubes were designed in which cells were planted on the wall of a tube inserted in a drum at a slight angle. The drums were slowly rotated, providing better aeration for the cells. In recent years the method has been modified by using large plastic cylinders which are rotated on special racks equipped with rollers. The purpose is to generate large amounts of cells. In these cultures the cells form monolayers lining the walls of the cylinders.

Petri Dish Cultures

Cells can grow on plastic which are specially designed for tissue culture purposes. This relates to the density of charges on the surfaces. Petri dishes designed for tissue culture purposes are coated with a thin layer of specially formulated plastic. The properties of the plastic prepared by various companies differ and variations occur from batch to batch in petri dishes sold even by the same company. Petri dish cultures are mainly used when bicarbonate buffer and CO<sub>2</sub> atmosphere are used to control the pH of the medium.

Flask Cultures

Originally, flasks were made from Pyrex glass but the properties of the surface of the glass were variable and changed with the washing procedure used. They were therefore replaced by plastic flasks in which the surfaces on which the cells are grown are coated with specially formulated plastic, similar but not necessarily identical to that used in tissue culture petri dishes.

Achorage-Independent Cells

These cells are usually grown in suspensions. Suspension flasks are available, ranging from very small ones, a few tens of milliliters in size, to several thousands of milliliters in size.

Cultures of this type can be expanded greatly by using fermenters or special installations several hundreds of liters in size. Such cultures are mainly used for harvesting secretory products of cells, e.g., monoclonal antibodies and various lymphokines.

CELLS IN CULTURE

Tissue and cells can be isolated from animals of any age, from embryos to adults. However, the younger the animal from which the cells are taken, the more vigorously the cells grow in culture. It is relatively easy to grow cells from embryos, but very difficult to grow cells from old animals.

Cells isolated from an animal and planted in a culture are referred to as a primary culture. The composition of cells in a culture is not necessarily identical to the situation *in situ*. The isolation of the cells, their disaggregation and culture conditions may exert strong selective pressures on certain types of cells so that some cells are preferentially selected over others. Primary cultures, therefore, are comprised of cells which are a continuation of the cell lineage *in situ* but not every lineage may be represented nor is the frequency relationship between cells of various lineages necessarily maintained.

When primary cultures form a monolayer, the cells can be

disaggregated by mechanical or enzymatic means and transplanted into new flasks. The procedure can be repeated over and over. Cells from cultures originating from a primary culture are referred to as a "cell line". Cultures after several passages are not necessarily identical to the primary cultures. The continuous passage may provide specific selective pressure and preferentially select a certain subpopulation of the primary culture.

Cells in culture may also undergo genetic changes. For example, the mouse genome is very labile in cultures and on passage, heteroploid cells form. On the other hand, the human genome is very stable and cells retain the diploid number. However, diploid cells usually have a finite life which relates to the number of divisions the cells undergo. This number is specific for each cell type. Human fibroblasts can double approximately 50 times. During this time the cells undergo senescent changes and eventually die. However, if cells in culture transform genetically, they avoid senescence, become rapidly proliferating cells and acquire tumorigenic properties. The consequence of passaging cells and forming cell lines, therefore, leads to cell senescence or cell transformation, and heterogeneity of cell populations. Prolonged culturing increases the chance of contamination with microorganisms, viruses or cells from other cell lines.

#### SUMMARY

The degree of tissue organization and suitability for experimental manipulation depends on the culture procedures used. Fragment cultures maintain the most tissue organization and cell lines the least. Cell lines can be more easily used for experimental manipulations than can fragment cultures. There are a number of tissue culture preparations which fall between fragment and cell lines as far as tissue

organization and ease of experimental manipulation are concerned.

In recent years, more attention is being paid to cell interactions. Attempts are made to simulate tissue function *in situ*. For example, it is possible to make spinal cord - spinal ganglia preparations, spinal ganglia - muscle preparations, or preparations for studying functional connections between neurons of various parts of the brain. It is possible to make tissue preparations that simulate the blood-brain barrier, consisting of endothelium and astrocytes grown on opposite sides of a membrane, or to isolate islets of Langerhans from the pancreas and maintain their functional state in cultures. We are now on the threshold of a new era in which various tissue culture preparations such as keratinocytes, various secretory cells and immature cell precursors will be transplanted into humans to alleviate a number of disease processes. Tissue culture has reached a degree of sophistication at which its possibilities are limited only by the imagination of scientists.

TISSUE CULTURE IN TOXICOLOGY

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*In vitro* toxicity testing is a large and complex subject. My presentation must therefore be limited to some comments on the use of tissue culture in toxicity testing and to report on some experiments performed in our laboratory. Tissue culture is a recent addition to toxicology research and testing; only a relatively small number of laboratories use it extensively. The role of tissue culture in toxicology has been outlined by J.W. Grisham and G.J. Smith in their review on evaluation of toxic responses in mammalian cell culture systems (1984), as follows: "The rationale for the use of cultured mammalian cells for analysis of toxicity rests on the fact that the actions of chemicals that produce disease and death in the animal are ultimately exerted at the cellular level; the goal of *in vitro* toxicology is to use systems of cultured mammalian cells as simple and manipulatable analogs of animals".

All living cells have certain structures and functions in common. Moreover, certain structures and functions may be specific to a cell type, tissue or organ. Therefore, tissue culture may be used to

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study either a general or a specific toxicity. Most studies done so far were directed to testing general toxicity because of the ease of culture and manipulation of well established cell lines. Well characterized cell lines originating from various tissues and from many animal species are readily available from the American Type Culture Collection, Rockville, MD. Several endpoints have been used for general cytotoxicity testing. Such endpoints include: decrease in cell numbers of DNA content per culture, inhibition of cell multiplication by incorporating tritiated thymidine into cell nuclei; inhibition of uptake of uridine by cells, which relates to induction of growth stasis; inhibition of attachment of cells to substrates; reduction of formation of cell colonies, which relates to decrease of cell attachment and/or cell multiplication; cell viability determined by dye exclusion tests; concentration of toxicant at which earliest morphological changes become evident; inhibition of synthesis of total cell protein; neutral red incorporation into lysosomes of viable, non-injured cells; and determination of viable cells by the use of tetrazolium salt (MTT) which is converted to an insoluble blue formazan product by living cells but not by dying cells or their lytic debris.

Assays of protein determination, incorporation of neutral red and use of tetrazolium salt are adaptable to automated scanning of multiwell plates, the measurement of optical density in each well and transfer of data to a microcomputer programmed to convert results to dose-response curves. It is possible to determine the concentration of a test agent causing 50% reduction in optical density and this endpoint can be used for comparative purposes. Using such computerized methods, a large number of tests agents can be analyzed in a short time. These assays, the neutral red incorporation assay in particular, are well reproducible and have been used with a wide variety of cell cultures of mammalian, including human, and fish origin.

The results of such assays can be compared by determining correlation coefficients in which the concentrations of test agents required to obtain the endpoint are compared directly, or determining the rank correlation coefficient in which the relative ranking of toxicity for a series of test agents are compared. The rank correlation coefficient is less affected by individual sensitivities of test cells and can be used more effectively for comparing assays done on various cell types and species as well as assays done on animals.

A number of principles determined in toxicity testing in animals are also applicable to toxicity testing in tissue cultures. For example, strong correlation has been shown between the cytotoxicity of metals and their softness parameters ( $\delta p$ ). There is also a relationship between the cytotoxicity and the molecular configuration and physical and chemical properties within a group of chemicals (QSAR) and between lipophilicity of the molecules and the degree of their cytotoxicity. These principles can be used to predict the degree of toxicity of a chemical of known physical-chemical properties.

In many instances the endpoints of toxicants determined by tissue culture methods have been compared to similar endpoints determined by using animals and in most cases they compared well.

In recent years, a concentrated effort has been made to develop an alternative method to the Draize test which is used to determine irritancy of chemicals on the corneas of rabbits. In this test the degree of inflammation is used as the endpoint. The attempt has raised a number of problems. In the Draize test several cell types and humoral systems are affected by the toxicants. It is a non-parametric test and is difficult to compare quantitatively from one laboratory to another. On the other hand, the usual tissue culture assays are based

on the use of only one cell type. It is probably reasonable to assume that to replace the Draize test with tissue culture tests, would require a battery of assays. The difficulty will be to validate the assays because tissue culture assays are much more precise and have better reproducibility than the Draize test.

Determination of specific rather than general toxicity is more complex when using tissue cultures and more sophisticated procedures are required. So far, tissue culture has been used for determining mutagenicity, cell transformation (carcinogenesis), and neurotoxicity. For more specific tests, specific cell types must be isolated from tissues or organs, the cells must be identified in cultures, and pure or enriched cell culture preparations developed. Even then, the cells are not necessarily structurally or metabolically identical with their counterparts *in vivo*. Therefore, development of specific assays requires considerable research.

In some specific testing, metabolic activation of toxicants is required. In many instances the test cells do not have the required enzyme systems for the metabolic activation of the toxicant. In certain situations, metabolic activation in tissue cultures can be achieved by addition to the cultures of S-9 rat liver microsomal enzyme preparation or co-culture with liver cells or other cells. Such tissue culture preparations for toxicity studies are still in the early stage of development.

In our laboratory, for the past three years, we have been studying the toxicity of a number of organophosphates and carbamates on neural cell aggregate cultures. The cultures are prepared from the brains of 17-day rat fetuses. The cells are disaggregated, then allowed to aggregate into small clumps. On culturing, the cells within

the aggregates differentiate into neurons, oligodendrocytes and astrocytes. The cells have specific, reproducible organization. In such aggregates neurons are mainly located in the centre, surrounded by neuropil with a few glial cells. The periphery is composed mainly of astroglia. We used such three-dimensional models of nervous tissue for acute and subchronic (14-day) toxicity studies. We used three endpoints: cholinesterase inhibition morphological assessment of the cells in the aggregates, and total protein per culture.

Dr. L.M. Segal in our laboratory investigated the effects of malathion, malaoxon, fenitrothion, carbaryl, and carbofuran on cholinesterase inhibition in neural cell aggregates. He compared the median inhibitory concentrations ( $IC_{50}$ ) of pesticides in cultures to median lethal doses (oral  $LD_{50}$ ) of the same compounds in rats and mice and found good correlation (.971 with rats and .965 with mice). Malathion, however, was not toxic in cultures, even in very high dose levels. However, when S-9 rat liver microsomal enzyme preparation was added, malathion inhibited cholinesterase. It seems that such preparations mimicked normal *in vivo* metabolic activation of the pesticide by the liver.

Dr. Segal also investigated interactions between various pesticides using neural cell aggregate cultures. The results, summarized in Table 1, indicate that some interactions were antagonistic, some additive, and some synergistic. An important finding was that subchronic (14 day) treatment of cultures with fenitrothion, even in dosages that did not inhibit cholinesterase, caused death of neurons and in higher concentrations, death of glial cells. Dr. Segal's studies indicated that organized tissue culture preparations such as neural cell aggregates, can be used to evaluate specific effects of a toxicant, e.g., the degree of inhibition of cholinesterase, and at the same time

evaluate possible side effects, in this case, the death of neurons and accumulation of the pesticide within astrocytes.

Tissue culture lends itself well to the study of toxicants to organized tissue as well as to developing systems. For example, the development of skeletal muscle of embryonic limbs in cultures and the development of whole embryos from zygotes in cultures, have been used.

The use of tissue culture in toxicology is still in its infancy but all indications point to its becoming a very important and indispensable technology in the field.

SUMMARY OF OP AND CARBAMATE INTERACTIONS IN THE ACUTE INHIBITION OF  
CHOLINESTERASE ACTIVITY IN RAT BRAIN NEURAL CELL AGGREGATE CULTURES

	MALATHION	FENITROTHION	CARBOFURAN
MALATHION	—	(+ S-9) ANTAGONISM	(+ S-9) SYNERGISM
FENITROTHION	(- S-9)	—	(+ S-9) ANATAGONISM
CARBOFURAN	(- S-9) ADDITION (NO SYNERGISM OR ANTAGONISM)	(- S-9)	—
TRIALATE	—	(- S-9) ADDITION (NO SYNERGISM OR ANTAGONISM)	(- S-9) ADDITION (NO SYNERGISM OR ANTAGONISM)

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THE USE OF ANIMAL ALTERNATIVES IN THE SAFETY EVALUATION PROCESS

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THE USE OF ANIMAL ALTERNATIVES IN THE SAFETY EVALUATION PROCESS

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Toxicology, in its broadest sense, is the scientific discipline that concerns itself with the deleterious effects of chemical and physical agents on living organisms. Thus, toxicology is a biological science, since the target of the aggressor agent is a biological organism. Interest in the aggressor—the chemical or physical agent—is merely one aspect of the discipline.

Alternatives to the use of live animals are used quite extensively in toxicological research. Experiments that deal with mechanisms of action, for instance, make use of the most modern biochemical and physiological tools available. The nature of the research questions raised determines the experimental tools to be used in laboratory experiments. Today, we see use of homogenates, cellular suspensions, cell cultures, subcellular fractions, etc. One must distinguish these laboratory experiments, however, from those performed in the safety evaluation process.

The "safety evaluation process" is a toxicological laboratory endeavor that has a very precise objective. These data determine how a chemical may be used by humans without producing deleterious health

effects. This process encompasses all the laboratory studies that are eventually used by government regulatory agencies to determine the toxic potential of chemicals destined for use by humans (as medication, as food additives, in the occupational setting, etc.).

In our society, we are constantly in contact, voluntarily or involuntarily, with a great number of chemical agents. Today, there is a great concern to assure the members of our society that this contact exerts little or no effect on the state of our health. Thus, toxicological findings are used extensively to establish the safe use and handling of diverse chemicals—as medication, in the workplace, in our food, and in the environment.

Toxicology is both a qualitative and a quantitative discipline. One of the objectives of the toxicologist is to describe as completely as possible the potential deleterious effects a chemical may possess. One could look at this phase as being qualitative, since it is an inventory of potential toxic effects. The quantitative phase is the attempt by toxicologists to define the exposure conditions to the chemical that will lead to the manifestation of toxicity in the target organism. Regulatory agencies rely very heavily on the quantitative aspects of the toxicologic profile to determine the safe use of chemicals.

The safety evaluation process is the toxicological evaluation by which toxicologists establish both the qualitative and quantitative aspects of the toxic properties of a chemical, so that regulatory procedures can lead to the safe use of the agent. The toxicologist must have adequate scientific information to extrapolate to the human use situation.

The actual tests carried out vary from chemical to chemical. However, there are some general approaches that are common to all. Since humans might be exposed to the agent occasionally or repeatedly, toxicological studies are performed when the material is given only once (acute toxicity) or repetitively (chronic toxicity). Since we are dealing with potentially deleterious, or even life-threatening, effects, it is obvious that these studies cannot be performed in humans; they must be carried out in animals.

The quantitative phase is by far the more important part of the toxicological workup for regulatory decision. It is this phase that leads to the safe use of a given chemical. Thus, the toxicologist must establish the dosage and exposure conditions in animals that will not result in toxic responses.

In the last 10 years, there has been worldwide pressure on the toxicological community to re-evaluate the procedures that are used to establish toxicological profiles. Toxicologists have made, or are currently making, changes to laboratory approaches. These changes occur when it is clear that the new procedures will not yield less valuable toxicological information. The best example of this is seen with two tests that are considered controversial—the  $LD_{50}$  and the so-called Draize test.

Toxicologists worldwide have taken the position that one must have some information regarding lethal properties of chemicals, but that the information need not be precise. Thus, the newer protocols use fewer animals and fewer species. The alternative approaches do not supply less valid toxicological information. (The position of the Society of Toxicology of Canada (STC) on the  $LD_{50}$  test is given in the text that appends this presentation).

The Draize test, designed to assess tissue or ocular irritation, has also been modified; the chemical concentrations have been lessened, and the length of the tests has been shortened. The tests are terminated when tissue irritation is first recognized, not when it is fully developed, thus, alleviating unnecessary suffering to the animal.

Subchronic (90 days) and chronic studies (greater than 90 days, usually 1 or 2 years) are designed to uncover potential toxic effects that manifest themselves only when the host receives the aggressor agent repetitively for long periods of time. Extensive examination of tissues, as well as biochemical changes are performed. These studies also establish the "no observable effect levels" (NOELs) used for calculating safety margins for human exposures.

In the area of carcinogenesis, the problem is more complicated. The lesion is not one that occurs rapidly after chemical exposure. Animals must receive the chemical under investigation chronically for relatively long periods of time before the neoplastic process becomes evident. For potent genotoxic materials given to rats, the malignancies may appear after several months of treatment. However, with other agents, the rats must receive the agents daily for the better part of their lifetime. Thus the current long-term bioassays used to uncover oncogenic properties of test chemicals require that they receive the substance for 2 years; the rats are then killed and the tissues examined for oncogenic lesions.

For a number of potent carcinogens, it is known that the oncogenic process is initiated because of an action of the aggressor chemical on genetic material. These agents induce cellular mutations thought to progress on to the neoplastic response. Short-term *in vitro*

assays have been devised where one examines the mutagenic properties of test chemicals in culture systems. Bacteria and other microorganisms are used to detect these mutations. The battery of tests available is now quite large. Unfortunately, none of these tests allows one to conclude that the mutagenic agent is also carcinogenic. Thus, the short-term mutagenic tests cannot be thought of as alternatives to the 2-year, long-term chronic rodent bioassay.

The short-term *in vitro* assays, however, can be used as screening procedures in the safety evaluation process. They are relatively inexpensive and rapid. Since they can identify chemicals that possess mutagenic properties, agents that are found to be positive in this battery become likely candidates for inclusion in the very costly, time consuming, long-term assays. In some situations, positive findings in a mutagenic battery results in abandoning interest in the chemical; further development of the agents for commercial purposes may be terminated. These batteries become important in the decision-making process. But it must be emphasized that these short-term *in vitro* tests do not measure carcinogenicity.

The quantitative objective of the safety evaluation process is to establish how the chemical can be employed by humans without causing harm. Procedures are well established for assessing so-called safe conditions of exposure (threshold limit values (TLVs) in the occupational setting, acceptable daily intakes (ADIs) for food additives and residues, toxic dose ranges for therapeutic agents, etc.). It is important to understand that this extrapolation from animal data to the human use situation depends entirely on the applicability of the biological response measured. One has to be able to translate the response seen in the laboratory setting to the type of exposure conditions that humans will experience. At some point the toxicologist must express

the extrapolation of the laboratory findings in terms of the amount of material ingested or inhaled. A safe "dose" has to be established. The jump from the cell to an intact living human is too large. For example, an *in vitro* aqueous concentration cannot be converted to an exposure inhalation level (ppm for 8 hours 5 days per week) or an ingested lifetime dose (mg/kg body weight). This is probably the major limitation to the use of non-animal alternative methods in the safety evaluation process. It is quite clear that extremely artificial systems, like cell cultures, are not capable of yielding the quantitative data needed to establish such "doses". Furthermore, relative quantitative potencies for a family of chemicals established in a cell culture system cannot be converted to human exposure conditions for the same series of chemicals.

Another major constraint to the use of alternative procedures is the fact that one has to demonstrate that a new procedure is indeed valid. Validation of the procedure is absolutely essential before one can envision its promulgation as a credible alternative to the use of live animals. Validation can only be established when one actually compares the predictive capacity of the new test system to the predictive capacity of the existing test system. Unfortunately, many individuals who encourage the development of alternative procedures fail to realize the importance of this step in the development scheme.

The toxicological safety evaluation process is interested in the frequency with which a given adverse effect is observed in test situations. How many animals are affected, what percentage of the group is affected? Thus, the size of the sample "n" is very important. With most cell culture systems or other *in vitro* test systems, where many samples from the same cell or tissue source are subjected to exposure with an aggressor agent, one must remember that the sample "n" for

statistical evaluation of frequency is not the total number of tests run from the same cell source, but the number of different cell sources used to derive the test cells. A cell source that delivers 20 test samples still yields only an "n" of 1 for statistical purposes when frequency is assessed. To get an "n" of 10, one must replicate the culture test 10 times using 10 different cell culture sources. Even with *in vivo* tests this problem can arise. For example, toxicologists now agree that when teratology is assessed, the sample "n" is calculated from the number of litters examined, not the number of fetuses involved; the material unit defines the sample "n".

The safety evaluation process is fundamental to regulatory decisions. I believe that government agencies, with good reason, will maintain a very conservative attitude regarding the introduction or substitution of newer methodologies, including alternatives to the use of live animals, when a regulatory decision on human safety is required. *In vitro* methods lend themselves to questions that are very precise. On the other hand, "Can the chemical be used safely by humans", in my opinion, is not a precise question and is not likely to be answered by *in vitro* testing alone. I see that alternative *in vitro* methods serve a more important role in the initial screening of chemicals to establish which ones require extensive testing in live animals. Decisions by private industry might be made on the basis of these tests (is it worthwhile pursuing a commercial goal for the chemical in light of the adverse effects observed *in vitro*?). Thus, use of live animals may be lessened. Alternatives to live of animals in the safety evaluation process should be viewed in this light, not one where we anticipate that they will replace existing methodologies.

## SOCIETY OF TOXICOLOGY OF CANADA

POSITION PAPER ON THE LD<sub>50</sub>\*HISTORICAL BACKGROUND

In the early part of the 20th Century, many medicinal agents in use were available as impure mixtures or extracts of biologically derived materials ("biologicals") rather than as pure chemical forms. It was often difficult to prepare uniform products by such processes, since the amount of "active" ingredient varied considerably from product to product. For several of these agents, the active *therapeutic potency* of the mixture could be correlated with the *lethal potency* of the mixture or extract. If one could calculate with precision the lethal potency of the material, one could indirectly assess the therapeutic potency of the same material. Effective therapeutic "dosages" for biologicals were often expressed in "units of activity" rather than in units of weight. Thus, quantitative methods were devised to assess lethal potency with precision, as a means of establishing standardization of biologically derived medicinal agents.

For statistical reasons, the median lethal dosage (LD<sub>50</sub>, the dosage estimated to kill 50% of the universal population of the species under test) was found to be the most accurate means of quantifying lethal potency. Furthermore, the mathematical precision of the statistically estimated LD<sub>50</sub> was found to be directly related to the number of animals that were subjected to each test dose and the number of dosage levels (yielding values between 10% and 90% mortality)

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\*Adopted at the STC Annual Meeting on December 3, 1985

utilized to derive the lethality dose-response data. Thus, the LD<sub>50</sub> was introduced in pharmacology and toxicology because of an important need in the estimation of potency of certain classes of medicinal agents.

A more general application of the LD<sub>50</sub> determination followed. The quantification of lethality became widespread. The LD<sub>50</sub> became one of the first quantifiable experimental tools available to the toxicologist. With such a tool, toxicologists could classify and compare chemicals according to their quantitative lethal potencies. Extrapolations to the potential dangers to humans due to acute exposures to relatively large amounts of chemicals were made on the basis of LD<sub>50</sub> data derived in animals. These determinations were carried out in a variety of species and by different routes of administration.

#### PRESENT SITUATION

One cannot discuss the utility of the so-called "LD<sub>50</sub> test" in isolation. The assessment of life-threatening qualities of chemicals is an absolutely essential component of the safety evaluation process employed for the toxicological evaluation of diverse chemical substances, such as medicinal agents, cosmetics, food additives, pesticides, chemicals encountered in the household or the occupational setting, chemicals encountered in recreation or hobbycrafts, and chemicals dispersed in the environment. The toxicologist determines the potentially adverse effects that such substances might cause when various living species are exposed to chemicals under a variety of conditions. The species of greatest interest is, of course, the human being, but it is important to realize that many other mammalian and non-mammalian species can be the biological target of concern.

The assessment of the lethal properties of chemicals is usually associated with the acute toxicity phase of the safety evaluation process. Both the dosages and the exposure conditions that lead to the lethal response must be established in properly performed toxicological assessments. If humans are likely to come in contact with a particular chemical (voluntarily or involuntarily, accidentally or by design), one must know where the lethal range exists, of these individuals are to be protected. The safe handling of potentially lethal chemicals depends on adequate knowledge of lethal dosages and exposure conditions. The design of treatment procedures or specific antidotes to be used in the case of chemical intoxications depends on adequate knowledge of the lethal process. Questions raised regarding the precision one needs when performing the " $LD_{50}$  test" are legitimate questions. On the other hand, questions dealing with the necessity of lethality assessment must be rational and in keeping with the responsibility of protecting society.

Large amounts of  $LD_{50}$  data have been accumulated; their utility has been questioned by a number of toxicologists. Toxicologists have deplored the misuse of the  $LD_{50}$  value as a kind of "biological constant". Variability is the rule in biology. This is also true when the biological response is death.  $LD_{50}$  values exhibit both interspecies and intraspecies variation. Furthermore, factors such as age, nutritional state and environmental conditions are known to affect lethal potency. Thus, the  $LD_{50}$  value, regardless of its precision, can never be regarded as a constant.

Toxicologists also realize that a precisely determined (in a statistical sense)  $LD_{50}$  value (with its 95% fiducial limits) is still only an estimate of the situation that may prevail in the population of species under test. In view of the well known interspecies variation,

is great precision really necessary? Toxicologists are questioning the need for precision in the determination of  $LD_{50}$  values.

Toxicologists can obtain significant information on lethal potency and the process leading to lethality without the calculation of a precise  $LD_{50}$  value (one with very small 95% fiducial limits). It is important that the animals given lethal or near-lethal dosages be observed closely to gain knowledge of the functional and pathological alterations manifested by the animals. Questions regarding lethal potency can be resolved by the use of less precise statistical estimates than the ones traditionally employed to calculate  $LD_{50}$  values. Methods that require fewer numbers of animals can certainly be used to estimate an  $LD_{50}$  value or to yield a reasonable estimate of the dosages that border the lethal range. It is doubtful that much meaningful knowledge is lost by the application of such techniques in the safety evaluation process. On the other hand, a more complete examination of the animals employed to estimate lethal potency is to be encouraged. More can be done to obtain more meaningful biological data from animals used in lethality studies.

Questions have been raised about the utility of determining  $LD_{50}$  values in a number of different animals species. It must be remembered that one of the goals of the safety evaluation process is to provide data where one can extrapolate the findings observed in laboratory animals to the potentially adverse effects that might be observed in laboratory animals to the potentially adverse effects that might be observed in humans, domestic and wild animals, or animals in captivity exposed to the same chemicals. If the lethal dosage of the chemical is found to be similar in several species, extrapolation of toxicity to humans is more secure. If similar toxicological effects are observed in several animal species, it is probable that a common mechanism of

action is involved in these species and probably will occur in humans as well. Thus, extrapolation to humans should be more reliable. However, if the lethal dosage is found to vary considerably in a number of different species, extrapolation to humans becomes tenuous. Such an observation indicates that the toxicity is species-related and that further investigations are needed to determine which species resembles the human. Thus, the determination of lethal potency in several species can have a marked influence on the confidence with which extrapolation to the human exposure situation is carried out. Furthermore, such results can have an important influence on the kinds of additional toxicological or biological studies that might be required to resolve the issue. Thus, it would seem unwise to restrict *a priori* the number of species that should be tested in lethality studies.

Important information can also be obtained from lethality studies performed with different routes of administration. In the past, such observations have had an important bearing on conclusions regarding the relative bioavailability (amount absorbed) of various chemicals following exposure by different routes of administration. They have been essential for determining how chemicals can be handled safely. These data can also help to establish the exposure conditions that are relatively without risk when chemicals are to be used as articles of commerce. Thus it would be unwise to limit *a priori* the routes of administration that should be employed in lethality studies.

Whether to employ a particular lethality test or not, or the precision one needs if the test is chosen, depends on the anticipated use that will be made of the data generated. This means that one must look at the toxicological questions that are being asked. Estimates of

acute lethal potency are presently very important data for the classification of chemicals when these substances are transported as hazardous chemicals. In the case of accidental spills and derailments, for instance, the adverse effects of consequence to humans are those associated with the temporary acute exposure to high concentrations of the chemical. In the occupational setting, accidental discharge may occur, resulting in acute exposures to potentially unsafe amounts. Acquisition of sound LD<sub>50</sub> data are essential in such situations.

It is important to point out that there are no known, validated alternatives to the use of animals for the assessment of lethal potency. Nor are such alternatives likely to appear in the near future. Attempts are being made to develop techniques that predict lethal properties of certain classes of chemicals on the basis of already known structure-activity relationships. Quantitative Structure-Activity Relationships (QSAR) and Quantitative Structure-Toxicity Relationships (QSTR) are examples of such approaches. The reliability of the QSAR approach depends on the availability of data reflecting (1) well-defined interactions between chemical substances (2) belonging to congeneric series of structures and (3) an already known active site in a biological system. The application of the QSAR approach is said to presuppose the presence of an active site coupled with unambiguousness (in terms of mechanism of action) of the observed biological effects. The present state of toxicological knowledge is far from providing the necessary data that could make use of the QSAR approach. Thus while these efforts are to be encouraged, it is evident that they will not be reliable substitutes for experiments in laboratory animals.

There is an important political issue that also bears on the safety evaluation process. Toxicological assessments are used to

protect the public from the potentially adverse effects of chemicals. Public perception is that individuals have the right to live in a so-called "safe" environment. The adversarial-litigation climate that reigns in North America reflects this public perception. This climate indirectly influences the practice of toxicology. What toxicologist or government regulator is likely to decide in favor of not performing a particular toxicological study, thought to be of limited value, when court litigation at some later date for this decision remains a possibility?

### CONCLUSIONS

The position of the Society of Toxicology of Canada on the issue of the so-called "LD<sub>50</sub> test" can be summarized as the following:

- a. The assessment of the lethal properties of chemicals is an essential component of toxicological evaluations designed to protect the public and the environment.
- b. Sound toxicological questions should determine the extent to which the evaluation of lethality should be pursued. The number of species tested, the range of dosages employed, and the number of routes investigated should be minimized but consistent with sound toxicological approaches.
- c. In most instances, high statistical precision of the LD<sub>50</sub> estimate does not appear to be essential. Consequently, procedures that permit the estimation of this parameter with a small number of animals should be the procedures of choice.

- d. All efforts should be carried out for the worldwide dissemination and communication of such results to prevent the unnecessary repetition of such studies.
- e. Toxicologists should contribute to the construction of biological data banks that may lead to the development of non-animal approaches to the estimation of lethal potency.

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ANIMALS OR REPLACEMENTS :

THE CANADIAN COUNCIL ON ANIMAL CARE PERSPECTIVE

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Dr. Harry C. Rowsell, Executive Director of the Canadian Council on Animal Care (CCAC), is a former Professor of Pathology of the School of Medicine, University of Ottawa, holding a DVM degree, a DVPH from the University of Toronto and a Ph.D from the University of Minnesota. In 1988, Dr. Rowsell was inducted as Officer of the Order of Canada. He has published more than 200 papers, abstracts, book reviews and book chapters, and prepared the section on Animal issues in the Canadian Encyclopedia. As a researcher in comparative medicine, his work involved observations on cardiovascular conditions using animal models of human diseases such as atherosclerosis and hemophilia. In 1968, working under the auspices of the Association of Universities and Colleges of Canada, he established the CCAC, becoming its first Executive Director. Its program has vastly enhanced animal care at research institutions throughout Canada. In 1980, the University of Saskatchewan conferred upon him an Honorary Doctor of Laws degree. As well, he is the first non-American to have been made an Honorary Member of the American College of Laboratory Animal Medicine. He has also received the University of Tokyo's Replica of the Red Gate, the Medal of the Academy of Medical Sciences of the USSR, and an Honorary Professorship from the Peking Union Medical College, People's Republic of China. He has also received an Honorary Membership in the Chinese Association for Laboratory Animal Science, Honorary Associateship in the UK's Royal College of Veterinary

Surgeons (in the UK) and July 8 will be made an Honorary Membership in the British Laboratory Animal Veterinary Association. Dr. Rowsell is now serving his second four-year term as President of the 40-national International Council for Laboratory Animal Science and is President of the Animal Air Transportation Association.

ANIMALS OR REPLACEMENTS:  
THE CANADIAN COUNCIL ON ANIMAL CARE PERSPECTIVE

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INTRODUCTION

Today's workshop on alternatives comes as a result of a proposal by the DRES/ACC that such a seminar would be of value to DRES employees and scientists. The Canadian Council on Animal Care (CCAC) became aware of the idea when the DRES/ACC asked Dr. Denny Madill, the Department of National Defence (DND) representative on the CCAC, to solicit the Council's advice concerning topics and speakers.

I welcomed Dr. G. Kimbell's remarks this morning, and the very close association we have had at this symposium. Dr. C.C-Mendoza has done yeoman service in making the arrangements for this seminar. I know he has been ably supported by his associates, and we are in their debt for this opportunity.

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Presented to Workshop on Alternatives to Animals in Research, Defence Research Establishment Suffield, Ralston, Alberta, September 16-17, 1987.

I have been asked by Dr. C-Mendoza and the organizing committee to discuss:

- a. The CCAC's position regarding the use of animals in laboratories;
- b. current trends in the use of animals in tissue cultures;
- c. ethical consideration regarding the use of experimental animals.

I cannot cover in detail all of this material because, in some instances, the information is not available to the CCAC. For example, we do not know the number of tissue culture systems in use in Canadian laboratories, or the answer to the often-posed question: "How many animals have been replaced through the use of alternative techniques?" We can, however, touch briefly on some of the anecdotal information which we have concerning the replacement of animals, primarily relating this to the use of animals in teaching. We have no access to crystal balls that would yield precise information on how many animals have been replaced through the use of techniques other than live animals for testing, particularly in toxicity testing. However, in future, we expect to be able to compile figures for specific areas of animal use through the CCAC's new computer program "Animal Research Protocol Management System". The program will be introduced this fall in Canadian universities.

#### CCAC SUPPORT FOR ALTERNATIVES

Since its formation, the CCAC has promoted the use of alternatives to laboratory animals (1). It was finally successful in 1982 in

persuading the Federal Minister of State, Science and Technology, to fund an annual course in tissue culture. In charge of the course has been Dr. Sergey Fedoroff, of the University of Saskatchewan, one of today's guest lecturers. Assistance in the conduct of workshops such as this is also one of CCAC's endeavours. The 1988 course to be held May 5-13, will focus on neurobiology.

As further measures of reducing animal use, the CCAC has established programmes in which information regarding availability of non-human primates and primate tissue is circulated, either through the CCAC newsletter, Resource, or by memoranda.

Another CCAC program is aimed at preserving unique animal models. One successful example of this practice is a colony of BB Wistar rats which are animal models for Type 1 Juvenile Onset Diabetes. The colony, which was in danger of extinction, is now being maintained by the Health Protection Branch, Health and Welfare Canada. This year, on the basis of studies in these animals, cyclosporin, an immunosuppressant drug used in transplant work, was found to inhibit development of diabetes in humans. Clinical trials have been conducted, and double blind studies are now under way. Dr. Calvin Stiller, of the University of Western Ontario, has led this research.

Canada's major grant-giving agencies have supported the development of alternatives. The Natural Sciences and Engineering Research Council (NSERC) has invited Canadian researchers to apply for grants for studies specifically aimed at developing alternatives to the use of laboratory animals. Both NSERC and the Medical Research Council (MRC) encourage the use of alternative methods whenever appropriate. Recently, they asked the CCAC to enlarge its Advisory Committee on Alternatives.

Canada has assisted in the promotion of programmes offered in the U.S. by the Center for Alternatives to Animal Testing (CAAT), located at the Johns Hopkins Medical Institutes, Baltimore, Maryland. "The CCAC", says CAAT's Director, Dr. Alan Goldberg, "has been at the forefront (regarding alternatives) and expressed itself to the scientific and lay communities clearly and very appropriately".

We have been assured by Government departments using animals that replacements are sought (and used) wherever possible. For example, Agriculture Canada is employing a new rabies test, in use in only three laboratories world-wide, that reduces animal use by 30,000 mice per year.

In Canada in 1986, 191,753 animals, 76% of them mice, were used in testing, according to CCAC figures. Very little testing of cosmetics or household products is carried out here, because of Canada's branch plant economy. What little testing is done is "mostly by Government, often in response to complaints (medical problems reported)", according to the Canadian Federation of Human Societies' "Safety Testing of Toxic Substances: A Survey" (2). This was reiterated by federal officials at the CFHS symposium on toxicity testing held May 12, 1987 in Ottawa (3). Nonetheless, ARK II and the Montreal-based Canadian Society for the Prevention of Cruelty to Animals, mounted a campaign against Gillette's testing procedures that generated 3,000 responses.

Current trends in the use of tissue culture in research are the venue of eminent scientists such as Drs. D. Ilse, G. Plaa and S. Fedoroff, for they are actually in the front line of that work. They know exactly what is happening. They know what the applications are, as you saw well demonstrated this morning.

OFFICE OF TECHNOLOGY ASSESSMENT REPORT

May I commend you for your interest "Alternatives to Animal use in Research, Testing and Education" (4). A publication of the Office of Technology Assessment of the U.S. Congress, it was published in 1986 and contains a great deal of valuable information. It discusses for example, current use of testing of non-living systems such as mathematical and computer models, chemical systems and epidemiologic data on humans. Although describing the last named as "perhaps the most useful alternative to animal testing", the document notes that a major disadvantage of such studies is that considerable human exposure can take place before a toxic effect is detectable; they are also expensive to conduct.

In describing the LD<sub>50</sub> test, "which many regulatory schemes rely on for classifying substances", the report notes political pressure to abolish it, the fact that extrapolation of humans is only rough, and that results vary widely between (and even within) laboratories. Other tests, including the Approximate Lethal Dose, developed in the 1940's, use far fewer animals; however, "although many investigators are moving to less precise LD<sub>50</sub> tests, no generally accepted alternative seems to have emerged", the report states.

In discussing the Draize (eye irritancy) test using rabbits, and skin irritancy tests, the OTA report says these methods have been criticized for the pain inflicted, poor extrapolation to humans (because the rabbit's eye and skin differ from humans) and poor reproducibility. It notes *in vitro* substitutions such as the chorioallantoic chicken membrane. Meanwhile, it suggests substitution of skin irritancy tests for eye tests, use of local anesthetics, use of more dilute solutions and testing whole eyes *in vitro*, using cattle eyes from slaughterhouses.

The report predicts that high costs of animals, public pressure and improvements in toxicological methods could bring a review of current legal requirements for testing, "perhaps reducing the amount of testing per chemical and the number of animals per test."

The report concludes: "Despite the problems of extrapolating to humans other shortcomings of animal testing techniques, the use of animals in testing is an integral part of the Nation's attempt to protect human health. Ideally, as the practice of toxicology advances, there will be less emphasis on numerical values in certain tests and more consideration of mechanisms by which toxic effects occur."

This OTA publication, in turn, has been discussed by England's Dr. Michael Balls, Chairman of the Board of Trustees of the Fund for the Replacement of Animals in Medical Experiments (FRAME) (5). "Contrary to what many in the U.K. have tended to think", he writes, "we have never known precisely how many animals were used in laboratories for purposes controlled by the Cruelty to Animals Act 1876. The annual Home Office Statistics of Experiments on Living Animals indicate the number of experiments started in a particular year and not numbers of animals (as is wrongly suggested in Table 16-3 of the OTA Report)".

"The position in the U.S.A is much, much worse, for institutions are under no obligation to record animal use, particularly when the work is not funded by a Federal agency," wrote Balls. "Thus, as this section of the Report points out, seemingly reasonable estimates of animal use for research, testing and education in the U.S.A vary from 10 million to 100 million per annum".

CCAC POSITION ON ALTERNATIVES

The Canadian Council on Animal Care's position on replacement of live animals was first enunciated in CCAC's original publication Care of Experimental Animals. A Guide for Canada, in 1968, at the time of the Council's formation, stated: "Before the decision to use an experimental animal is made, consideration must first be given to the possibility that lower orders or non-sentient methods would provide the necessary information. If not, it is the responsibility of the investigator to choose judiciously the species of his experimental animal" (6).

The present Guide recommends that the research, teaching and testing communities should "avoid unnecessary (animal) use". It cites also Russell and Burch's "Human Experimental Techniques", including their now famous "3R principle" Reduction, Refinement and Replacement for animal use (8). Later, I added another proposal that people who are using animals should use "the right animal for the right reason" (9).

As a veterinarian, and one whose profession is dedicated to the relief of pain and suffering in animals, I can now take the position that, given the development of new techniques and new technology, we have made steady advancement since 1968 in all of the fields of animal use. Now I would like to see us pay strict attention to the use of any of the possibly endangered species and non-human primates. As well, we must aim toward a lessening of the invasive studies that are commonplace in some countries. Unfortunately, there are still some students being taught veterinary surgery using multiple invasive techniques and; not really having sufficient concern for the prevention of pain and suffering in their animal subjects. Therefore, we have some

housecleaning to do in our own profession; however, I would hope that what we would learn from this symposium would be that there are many ways of replacing animals.

In Canada, 18 species of animals are used in research. A significant advance that has occurred during the lifetime of the CCAC, the Canadian Association for Laboratory Animal Science (CALAS) and the International Council for Laboratory Animal Science (ICLAS), is that significant progress has been made toward producing healthy, quality animals and accepting the need for such animals: animals of genetic and microbiological definition (10). We are no longer dependent upon the "backyard breeders"; however, the need for reliable sources of animals is still something we have to address.

For the rodents and guinea pigs, both genetic and microbiological quality controls are of immense value. It has been demonstrated that genetic quality can affect scientific results. Rabbits are now attaining the status of Specific Pathogen Free, as are some cats. The use of random source dogs and cats is being questioned, as purpose bred mongrel dogs and cats are increasing in supply. However, there is a use for random source animals in acute, non-survival studies. One controversial issue is the use of random source dogs in chronic, long-term surgery, some of the physiological studies and the neurosciences, where possibly a better defined and a purpose bred animal would be and should be used.

There has been a great decrease in the use of non-human primates and their capture from the wild comes under ever-increasing scrutiny, both by the scientist and the concerned conservationist. Many monkeys now are derived from primate breeding programs. In Canada, the largest such program is that of the Health Protection Branch of Health and

Welfare Canada. Its breeding colony for cynomolgus monkeys is located in Ottawa.

Laboratory animal science is not stationary. Animal facilities, equipment, caging and the animals themselves have changed significantly since biomedical research mushroomed post World War II. Similarly, changes have occurred in the requirements for technicians and the scientists caring for or using animals in research (11-15). These changes have greatly increased the cost of biomedical research. Coupled with the foregoing change has been an increase in, and a demand for, the examination of the ethical issues surrounding the use of animals in research (16-19). However, of paramount interest has been the requirement by the scientist for obtaining valid scientific results.

One of the new requirements forthcoming for rats will address some of their behavioural needs. If one thinks about it, mice have always been able to stand on their hind legs and extend their heads in their cages; whereas, rats have never been able to do so. It is expected that new recommendations will make allowances for behavioural abilities and allow rats the opportunity to stand up and be investigative. Such behavioural enrichment will be of increasing importance in the coming decade. Looking after psychological well-being will become the duty of those using animals. We have much to learn. We used to think that if we converted a room to animal use and filled the cages, that was perfectly all right. However, we have come a long way in 20 years toward discovering the many many factors that affect the animals, and CCAC has established a Committee to address this issue.

In addressing the issue of animal well being, there are many environmental factors which must be considered (20-22); physical

(temperature, humidity, lighting, noise), chemical (food water air, insecticides, miscellaneous chemicals) as well as microbial factors which have some interrelationships with physical and chemical factors (population per cage pathogens, ammonia levels, and bedding). Thus, it is clear that, in addition to stress (23,24) virtually all factors in the environment may affect significantly the biologic responses of experimental rodent.

A variable, which we considered had been addressed, is ventilation rates of animal rooms; however, the importance of air distribution delivering an even proportion of fresh air to every cage in the room is now being stressed. The CCAC ventilation requirements are 12-15 air changes per hour. Depending on the design of the air distribution system, it has been shown that the efficiency of air change varies from 32-95%, with actual ventilation rates varying from 4-15 air changes per hour.

#### ANIMAL USE

It is important to divide the areas of animal use: research, teaching, and testing. Testing is the most difficult area with which the Council must deal. While the CCAC can influence the areas of research and teaching, we cannot greatly influence the use of animals in testing. Dr. Plaa has given many good reasons why the regulators are not going to change from entire animal to *in vitro* systems unless there is validation of such techniques. However, it is important to develop communications with the regulators, and the CCAC has done so. Government departments are using many *in vitro* techniques, both in research and in screening procedures; however, in regulatory safety testing, the use of animals remains.

It is hoped that the scientist will examine the use of animals with sensitivity, asking the question: Does the end justify the means? Is what is going to be gained from the study beyond humanitarian concepts and acceptability? There are well-trained technicians, many in the veterinary profession, who can assist the scientists in achieving the 3R Principle. Improvements to protocols and the more judicious use of animals has to be by consensus and consultation, and not by force or by "police action" on the part of an Animal Care Committee, or the veterinarian. It has to be done in concert with and with respect for each other's position.

#### PUBLICATIONS

The CCAC Guides, with which I hope you are familiar, are now being revised. We intend to combine Volume 1 and Volume 2 and we intend to produce a small vade-mecum as a standard guide on general principles, which will be a source for immediate information concerning the care and use of animals.

The CCAC publishes a semi-annual newsletter, Resource. If you are not on the mailing list we invite you to so request. Resource contains information about new non-animal testing that is being conducted and we try to keep abreast of that type of information.

American scientists refer to the Guide to the Care of Laboratory Animals, produced for the U.S. Public Health Service, distributed by the Institute for Laboratory Animal Resources (25). Although it is not quite as extensive as our Guide, it is a very valuable addition to one's library and it is a requirement of all grantees of NIH to follow. In the U.K., the 6th Edition of the Universities Federation for Animal Welfare Handbook has just been published (26). International guiding

Principles for Biomedical Research Involving Animals, have been published by the Council of International Organizations of Medical Sciences, (an arm of WHO) (27).

The issue of the use of alternatives *per se* is discussed quite widely in the literature (28-34). The newsletters of FRAME (35) and CAAT (36) are perhaps the most useful regarding alternatives. In Canada, in addition to Resource, the CFHS publication, Caring for Animals, informs Animal Care Committees of such advances (37). FRAME's journal, Alternatives to Live Animals (ATLA), is extremely helpful.

While the American scientific journal, Laboratory Animal Science, does not contain much information about alternatives, it does on occasion, discuss refinement techniques. For example, regarding the use of Freund's adjuvant and its possible replacement, which is one of the CCAC's concerns, Niemi has discussed replacement techniques which cause less pain and suffering in the animals (38). Osebold has also discussed this topic in the Journal of the American Veterinary Medical Association (39).

The Federation of European Laboratory Animal Science Association's (FELASA) journal, Laboratory Animals, which carries technical information, also carries information about refinement. The bulletin of the Institute for Laboratory Animal Resources in the U.S. from time to time also includes information about alternative techniques. The international Council for Laboratory Animal Science Bulletin is also recommended.

There are, as well, many books that deal with replacement techniques. An excellent example is "Of Mice, Models and Men" by Andrew Rowan (40). Rowan, who has agreed to sit on the CCAC's Alternatives

Committee, is the former executive director of FRAME, then associate director of the Institute for the Study of Animal Problems in the United States, and is presently assistant dean of new programs, School of Veterinary Medicine, Tufts University, Boston. There are also publications resulting from workshops such as the Scientists Center for Animal Welfare's Effective Animal Care and Use Committees. One of the workshops in the series that produced them was held at the University of Toronto and a consensus document on protocol review resulted from these meetings (41).

Even the popular press contains articles on alternatives (42). Dr. Ilse and Dr. Plaa have in their discussions reviewed the place for computer systems. McMaster University has done some interesting work with physiological models (43,44).

#### CURRENT ATTITUDES

Organizations, such as the International Association Against Painful Experiments on Animals, in the past have been very supportive of responsible use of animals, seeking ways and means of decreasing those experiments that cause pain. Certainly, alleviation of pain and suffering concern scientists and animal welfarists alike (46-55).

Conversely, there are other organizations that are unalterably opposed to the use of animals in research. For example, Lifeforce, an organization on the West Coast, even opposed the raising of money by Rick Hansen for spinal cord research (56). Another antivivisection group is the radical Animal Liberation Front. These individuals quote urban guerillas, vandalize research laboratories, and release animals (57). The CCAC does not defend individual research. It does, however,

defend the system that we have in place to control the use of animals in research. We need not react each time an antivivisection organization charges "animal cruelty".

Another topic which has concerned the CCAC is factory farming (58,59). A very excellent book, "Should Trees have Standing?" by Christopher Stone (60), was written in response to the Sierra Club's desire to save a mountainside of trees that Walt Disney Studios wanted to tear down to make a new film. Through those efforts, the project was stopped and the trees saved. Therefore, there are many causes and protection groups.

The Research Defence Society in the U.K. is not so much like our Canadians for Health Research, but not quite the unbridled advocate of the researcher's rights as the national Association for Biomedical Research (NABR), a prestigious British society. It contacted Sir William Paton, professor of pharmacology at Oxford University to write the book, "Man and Mouse. Animals in Medical Research" (61). This is a very well balanced publication. Sir William recognized that some scientists would dislike his efforts and some in the animal welfare community would claim he is incorrect; however he puts the case for the responsible use of animals in experiments very capably.

There are many other articles that appear from time to time, and it is one of the responsibilities of the Council to keep this information before the scientific community. We publicize new publications in the Resource as much as possible. We recognize the complexity of the area with which the Canadian Council on Animal Care deals. It is a very mixed community. We have many scientists out there who believe in their own autonomy and feel they should be allowed their own selections (of methods, animals) without interference. We recognize that there

has to be freedom of the individual to pursue independent lines and enquiries, but working through, and this is the important thing, certain objectives: e.g., informed and explicit peer judgment. Listening to others is extremely important. The lone cowboy on the range is disappearing very very rapidly. We, in the research community, have our undisciplined cowboys as well and these we have to educate. At the recent XXIII world veterinary congress, an entire plenary session addressed "The use of animals. A necessity and a responsibility". You have that responsibility.

I cannot stress more strongly the emphasis that CCAC is placing on the local Animal Care Committees, in protocol review, to require investigators to see whether there are ways to achieve their scientific goals in a more humane way and, I might say, in most cases, more economically.

#### TOXICITY TESTING

There continues to be a great deal of testing necessary in animals. part of this is to satisfy the consumer. We heard this morning that "What the consumer wants, the consumer gets". We have to realize that one section of society demands protection for consumers and another protection for the animal. At the moment, I think the consumers and their demands are taking precedence over the animal protectors. However, some, in the consumer groups, are now listening to what is being said by others.

Toxicity testing is one area which in future may require far fewer animals as non-animal tests are introduced (62, 63). Unfortunately, even the non-animal tests often use animal products. Moreover, they require validation before they can be substituted for animal use.

Two examples of alternatives are the Limulus Amoebocyte Lysate test for pyrogens, which uses horseshoe crab blood as a substitute for live rabbits, and the Ames test using *Salmonella*, a bacterium. The public has soundly criticized use of the LD<sub>50</sub> where half the test animals die, and the Draize (eye irritancy) test in rabbits. Regarding the Draize test, as a veterinary pathologist, I would like to know why the test could not be stopped at the point when the eye shows inflammatory change, rather than letting the process continue to ulceration.

There is also a restriction to using strongly acidic and caustic materials in the Draize test. The head of the consumer products testing in the U.S., when asked the number of the cosmetic agents tested which produced a positive Draize test, replied that it would be less than 0.1 percent. Before the product is introduced into rabbit's eye, other *in vitro* tests have been used.

Regarding the LD<sub>50</sub> test, the Lancet, a prominent medical science journal, published an advertisement for an antivivisection society opposing the LD<sub>50</sub> test. Our own Canadian Federation of Human Societies wonders "The LD<sub>50</sub>: when will it die?" (64). As far as Canada is concerned, The Canadian government is one agency that has never really required or demanded the use of LD<sub>50</sub> test. It should be noted, however, that Health and Welfare Canada Minister Jake Epp considers that even though the government conducts LD<sub>50</sub> tests itself, "its determination is an important step in the acute toxicity assessment of any new substance to which human beings may be exposed (e.g., "poison.....content."). "We must, therefore, continue its use until such time as an alternative is found," Epp says (66). Dr. Plaa mentioned this morning that few toxicologists use the LD<sub>50</sub> test. This is also the case in the U.K. (63).

The International Coalition to Stop Use of the Draize Test and the LD<sub>50</sub> is headed by Henry Spira, who, at a meeting in Washington, said: "If you want help to find alternatives to animal testing, go to the scientific community, because the scientific community is going to provide the help; they do have the leadership, they do have the interest and you cannot find people who will work into this area and you are not going to find that kind of help in the animal welfare community.

In the U.K., some seven universities are now attempting to validate the various procedures, and FRAME is cooperating with Johns Hopkins Center for Alternatives to Animal Testing, headed by Alan Goldberg, along with Denis Stark. Rockefeller University and other universities are trying to develop a network that will do validation and testing. However, as Dr. Plaa mentioned, it is still a long way down the road.

Last year, Johns Hopkins presented a symposium, "Approaches to Validation". At that time, a representative of the Office of Technology Assessment (OTA) reviewed many of the things that Dr. Ilse mentioned concerning the difficulties associated with getting the regulating authorities to change the test requirements. His assessment was: "Don't expect that you will have the validation tests and requirements that the alternatives be used by the regulatory agents perhaps for 10 to 15 years". It takes a long time until such agencies change their requirements. They must be very sure, because it is they who must shoulder the blame if something goes wrong.

Johns Hopkins, November 4, 5, will hold its Fifth Annual Symposium entitled, "Progress in *in vitro* toxicology, in Baltimore. CCAC tries to attend such meetings, and to learn what is happening in the field, and to attempt to keep abreast of this very complex issue.

My own area is the study of atherosclerosis. I used to say: "There is no way we can do anything in tissue culture in the study of atherosclerosis. You have to work with the whole animal." While we now know this was an incorrect conclusion, it was valid at the time. However, now we can demonstrate in tissue cultures that pathological changes involved in the formation of atheroma and we can examine specific changes and various factors involved: things that we used to think were only possible in *in vitro* situations.

The CCAC is concerned that we address, in the Ethics of Animal Experimentation monograph, the end point of *in vivo* studies. Where does the end point come? When you're doing a Draize test, where does the end point come? Where does the end point come when you're doing a toxicity test? CCAC considers barbaric any requirement that such studies continue until the death of the animal. I think Council's statement in this regard is the most important statement that CCAC has to address in its Ethics statement at the present time. It is "Studies such as toxicological and biological testing, cancer research and infectious disease investigation may, in the past, have required continuation until the death of the animal. However, in the face of distinct signs that such processes are causing irreversible pain or stress, alternative end points should be sought to satisfy both the requirements of the study and the needs of the animal."

The Animals for Research Act of the Province of Ontario came into effect because it was felt, at that time, that they could not train medical students unless they had dogs for their physiology labs. There are very few physiology labs today that use any whole animals. Even in the veterinary schools they are being reduced in numbers: not because the studies are cruel—most of them were acute, non-survival—but because of the time spent and the value gained: they were not very effective.

There are other problems that will have to be faced down the road. Utilization of human DNA will create a disease model that will be identical to the human disease; we will have duplicated in an animal the precise model of a human disease. This genetic technology will be questioned on the basis of the ethical issues and how far we should allow biotechnology to go. There continues to be requirements for dealing with all animal tissues, developing a balanced, rational decision. A great deal of research must be continued both *in vivo* and *in vitro*. Prevention of suffering both in man and animals is a major objective. Hopefully, through *in vitro* techniques, we may reach this goal more rapidly.

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UNCLASSIFIED

WORKSHOP DISCUSSION

UNCLASSIFIED

QUESTIONS AND ANSWERS

Dr. Paul Lundy: You mentioned this morning that perhaps it was not necessary to get a definite figure on the  $LD_{50}$  when using some of the newer methods. For example, I wonder, in terms of editorial peer review, where this situation lies right now? Do journals of toxicology accept data which cannot be compared very easily without the usual limits. Without an exact  $LD_{50}$ , it is hard to claim that one drug works better than another drug -- for example. I think it very unlikely that a prominent journal would accept that sort of data.

Dr. Plaa: What is the question that you're asking? If the question that you're asking is in terms of antidotal therapy, and that's the point that you're trying to develop, and you're going to say that this one is better than the other one, and you have to use the lethal effect as your end point, then I think you are obliged to go through the regular  $LD_{50}$  with the narrow fiducial limits, (and establish) an idea of the slope, because you are comparing two dose response curves. Therefore, if that is the object of your point, you would have a great deal of trouble getting this material admitted into, for example, Toxicology and Applied Pharmacology, or Fundamental and Applied Toxicology. If it has not been well characterized, you haven't done it really properly.

However, if you were only using the  $LD_{50}$ , as you're testing against doses of multiples of the  $LD_{50}$ , what difference does it make whether your  $LD_{50}$  is really very precise, or whether it's not, because you're using the very same factor throughout. I could see in that situation that you could get by, probably, with an estimate of the  $LD_{50}$ , by the

"up and down" method: The Brownley method. You can use a minimum of six to 10 animals. You are bracketing your  $LD_{50}$ , and you will get a value. And there is also the "Thompson and Wile" method where you have fixed numbers, and, by tables, you get some sort of fiducial limits. You can use those sparingly because you are just ball-parking a dose. And then you say "multiples of that" and you are using your test compound and it would be all right. However, if you are actually going to compare the dose response curve under one set of situations, to the dose response curve in another situation, I think you are forced to go through the procedure. It is going to use an awful lot of animals, and there will be a lot of data points in between. What I was talking about was in terms of acute toxicity studies in the safety evaluation process. The  $LD_{50}$  has very little utility for extrapolation about the real toxicity of this material to humans.

It is a ball park figure, but it is not one that a lot of other things are going to depend upon. However, in your situation, that  $LD_{50}$  is important, and I could envision that you could end up having a situation in which it is the dose response, not so much the  $LD_{50}$ , that is modified by your treatment. You wouldn't modify it very much -- but you might modify the slope of that line, in which the dose response either becomes flatter, or it becomes much more vertical. And there, I think you are obliged to go through. That, I say, is a research type of  $LD_{50}$ . It is not just a routine  $LD_{50}$  that is used for regulatory purposes.

Dr. Lundy: Would we come under criticism by the animal rights people for doing that sort of thing, although it has been a pretty standard scientific practice and necessary to obtain the goals of the research?

Dr. Plaa: I don't know. Maybe Dr. Rowsell would like to answer.

Dr. Rowsell: The CCAC has heard all the arguments against the value of the LD<sub>50</sub> in the safety testing of drugs, and yet, we know that there are situations in research where it is required. We have directed the Animal Care Committees to ask the investigators to justify the need for the LD<sub>50</sub> in their research should this be included in a research proposal. On some occasions, we have had researchers who have decided that they could use a limit test; they could use fewer animals. However, one of the very outstanding examples was an investigator who was looking at *E. coli* endotoxins. He held a prominent position at the university; however, he appeared before the ACC and defended his need to use the LD<sub>50</sub> in this research situation. Regarding the animal rights groups, you have to realize that most are abolitionists. In this particular instance, there was a representative of the local humane society on the ACC, and she, as a layperson, was convinced, as was a philosopher on the Committee, by the statements of the others on the ACC, that the use of the LD<sub>50</sub> was necessary to the research.

Dr. Plaa: I would want to know how the animal is to die. If the animal has to go through a series of convulsive episodes before he dies, I would be leery of saying: "You're going to keep that thing going, until the animal is dead". I would say: "Is it not possible, since you have shown the relationship between the onset of convulsions and eventual death, why not call the onset of convulsions your end point? Stop it there".

Dr. Rowsell: CCAC's ethical guidelines call for finding different end points. I understand through Dr. Zbinden in Switzerland that the process they've been following for many years in that country is that when there are signs of impending death or severe pain or distress that cannot be relieved, the animal is killed using a humane method.

Now I have heard people in the drug industry claim, and perhaps Dr. Ilse could confirm this, that if you kill the animals too soon, you won't see the effect on all the target organs; so that the animals have to die. I don't know enough about it, but that is the other side of the coin.

Dr. Ilse: One is an acute toxicity issue. The other is a longer-term toxic side effect issue. I know what Dr. Rowsell is asking: "Do you really have to keep your animals alive for 15 months, two years, while they might be suffering?" The regulatory agencies say: "Yes, because tumors don't really start to show themselves before about 18 months into a two-year rat study". When rats get to be about two years old, they are the equivalent of a 70-year-old human being in biological age, and tumors start showing in the rats only at about 18 months. So what you like to do in a tumor study is to plot the time of onset of the tumor. You get rats dying along the way, and you use them as milestones, and plot the onset of the tumor. That allows you then to forecast, as much as you are allowed to extrapolate from rat to human, what the risk is likely to be with your human population taking the drug. You say, are the tumors due to the drug, or are they spontaneous? So that is why you keep your animal alive as long as possible.

On the other hand, if I have a rat that has a huge, fibrous sarcoma that weighs almost as much as the rest of the rat, I am not going to keep that rat around. It is a matter for you to decide. It is the ethics that you wish to promote that govern your actions. And whether the FDA likes it or not, I will sacrifice that rat, and document it adequately.

The other issue is an acute toxicity. And, as Dr. Clark told you this morning, beautifully, the maximum tolerated dose (MTD) is something of

greater value to people in drug development, than is the  $LD_{50}$ , because, if you take your animals and you dose them with successively larger doses, and you see a number of increasing effects, or a greater variety of effects occurring as your doses are increased, that gives you much more information on the toxicology of the compound, and on the sequence of effects that we are likely to see later on in the drug development program. It also warns you of how much drug is likely to cause death when a child, say, takes a whole bottle of tablets; or, if some crazy woman takes a whole bottle of sleeping pills, what is likely to happen. So, I think the MTD, from my point of view, is far better than an  $LD_{50}$ .

Dr. Murray Hamilton: I think our concern here, at least mine, is that I have something that I know is lethal. I am not looking for what its actual chemical toxicity or signs are. What I am looking for is an antidote. I am looking for evidence. I am looking for a drug that I think is safe. And I want to compare how good it is, against something else. I would be interested in knowing how a Maximum Tolerated Dose, or a Minimum Lethal Dose could be used to do that, in a manner that is acceptable to my peers.

Dr. Plaa: Going along with what Dr. Rowsell is saying regarding end points, I think in that situation you would have multiple end points, anyway. Say you are talking about chromonestran subligitans. Now, I think that what antidotal treatment does to chromonestrates in addition, in itself, regardless of whether the animal dies or not, that is a series of things that you are interested in doing in comparison. But even if you have all of these other end points, I think you are still stuck at a certain point in time, that you may have to show that that antidote does protect the animal, and he does not die. And the only way you can say he does not die is that you measure in a situation where accurately he DOES die. You have to compare the absence of death

or reduced death, to a group that has died. But, in that situation, you are not doing LD<sub>50</sub>s just out of idle curiosity. You are doing the LD<sub>50</sub> because you are searching for a criterion that is going to alleviate.

On the other hand, if you went ahead and tangled with that same manuscript, and then you came out and you put in the LD<sub>50</sub>s in say, five different strains of mice, and 25 other species, and you wondered "What is this in here for"? "Of what utility are these other things?" Rather than just being curiosity, that is, I think, the judgmental point.

Question: Regarding end points, what if the product, which is a chemical that is supposed to be acted upon by the therapeutic drug, has a known (LD<sub>50</sub>) provided and what you call the "up and down" value is already known. The historical value is understood.

Dr. Fedoroff: "up and down" values. You already know that given historical values, an an accepted LD<sub>50</sub>. You don't have to go to LD<sub>50</sub>s all the time for a known toxin.

Dr. Plaa: But you probably, at some part in your experiment, will want to make sure that the material you are using is good, so you do sort of a preliminary estimation to make sure you're in the right ball park.

Dr. Fedoroff: What if, at the same time, you have 50 animals for analytical, too.

Dr. Plaa: I must add that I like an "up and down" approach. We used it, I wish I could say because we wanted to conserve dogs -- but it wasn't. It was really much too costly to use dogs, and we were

measuring the relative potency of liver damage and kidney damage with allo-alkanes and we wanted to compare species, and we wanted to do dogs. But we needed a reference point. We needed to find out what it was; how the liver injury would occur, relative to an effect on the Central Nervous System. And the best thing we could use on the CNS was actually death by narcosis. So, to do dogs, what we did was run the "up and down" method. But we showed in mice was that the "up and down" method would give us an  $LD_{50}$  which was not very far from the  $LD_{50}$  that we would run by the traditional method using lots of animals. So it is a very useful technique.

Dr. Hamilton: How do you handle animals that you know are going to die. For instance, very sick animals unable to eat or drink, say, in a 5 day  $LD_{50}$  determination. I'm inclined to terminate them, but will journals accept that intervention?

Dr. Plaa: Well, what you actually did in there was you selected animals which had died, or were moribund. And you included them. Today, that would go into the journals. I would say 10 years ago, that would have been a "no-no". Today I think you could explain that, and you can get it in. Editorially speaking, toxicology journals are very sensitive to inhumane treatment of animals, or anything like that. When I was editor of a toxicology journal, I spent most of my time making sure that the methods described included nothing that could be attacked on the basis of inhumaneness and cruelty. So they are very sensitive to the position that you have described.

Question: Regarding the  $LD_{50}$ , ... do you think the multiples of 2 or 3 animals at 2 or 3 doses are significant? I do not think we have to worry about how accurate these would be statistically. Even  $LD_{50}$ s can vary that much.

Dr. Plaa: I would say, if you have a rough idea of the  $LD_{50}$  or a very precise idea of the  $LD_{50}$ , you have exactly the same factor in both sides of the equation. Your rough estimate has been used in the two experiments, or your very precise estimate has been used in the two experiments. So the factor is already in there. It may make a difference; so if you are dealing with two, and you are talking about potency ratios, now you may have trouble. If you want to compare actual dose response lines, take the  $LD_{50}$ , and all of the other stuff is going to come into that picture.

But there are ways around that. You do not have to compare products only by comparing the total dose response line. If you are looking for protection, you pick a dose that does produce death, and then you see if you can not produce death. If you are looking for potentiation you do the reverse. But you can pick spots on that line, and get statistically-valid comparisons.

Question: What do you consider an acceptable way to cause the death of the animal.

Dr. Rowsell: I have been asked many times for my definition of humane euthanasia or humane killing, when one is taking the life of an animal. CCAC's position is that it is the production of a death that does not create additional pain, distress or suffering. However, all of these latter terms are subjective and must be examined by the individual's own experience, and, I would emphasize, their conscience.

However, one thing that must occur in order to produce this humane death, is an immediate depression of the Central Nervous System, so that the animal is incapable of feeling any additional pain. We do that through some methods that are open to question. One of these was

raised recently by the American Veterinary Medical Association Panel on Euthanasia. Its 1986 report says that decapitation may be painful in rats, based on a couple of studies that have been done but that have since been criticized by the neurosciences. The AVMA is now rethinking that particular position.

The CCAC position and that based on experience that I have had on euthanasia, is that in rats that have been implanted with electrodes, either in the reticular substance, the hippocampus or the lateral thalamic nuclei, which are supposedly areas of consciousness, the blinking reflex disappears immediately in the decapitated rat. As well, the EEG goes flat as quickly as it does when one administers an intravenous injection of pentobarbital sodium. Therefore, on that basis, the CCAC position remains that decapitation in the rat is an acceptable euthanasia method. It is also very important that the person who carries out the euthanasia procedure be most careful about not causing the animal undue stress.

Again, there are certain methods that the CCAC will not accept in the larger animals: decapitation of monkeys without light anesthesia; decapitation of cats or rabbits. We draw the line there because of the problems that occur in the flattening of the EEG and also with the blinking reflex, as well as the fact that there is still quite a blood supply that remains.

Recent controversy has developed over the decapitation of reptiles and amphibia. In reptiles, particularly, this is not an acceptable method.

Therefore, you have to carefully select your methods, and you have to select the person who will carry out the euthanasia.

In Ontario, more recently, all the anoxic methods for killing dogs and cats will be outlawed. These include nitrogen flushing, the use of carbon dioxide and carbon monoxide, and the use of high altitude decompression.

The field of euthanasia is under constant review. The best document, with the exception of the clause regarding decapitation in rats, is the 1986 Report of the AVMA Panel on Euthanasia, copies of which we can send to you.

Dr. Merle Olsen: I have found in many physiology and toxicology scientific journals the Materials and Methods sections are often very scanty about describing the animal experiment. And also I very, very rarely, almost never see a statement regarding animal use included in the article.

Dr. Rowsell: The CCAC has written to the majority of journals in Canada and asked them to include in their requirements for publishing that the author has followed the guidelines of the CCAC, and the submission of the manuscript is evidence of this. We do not ask the Journals to make an additional statement to this effect. I agree with you, Dr. Olsen, that it would be nice to have it; however, given current costs of publishing, and the fact that many of the authors are being charged page costs, we feel that if it is in the guidelines for the submission of manuscripts, that this is sufficient. I must say that in Canada we have the full support of the journals.

Dr. Richard Bide: How do you correlate data from the in vitro test in culture to the whole animal?

Dr. Fedoroff: As was mentioned by several, this is one of the most

difficult things there is, because there is not a really good, fool-proof method to correlate results from tissue culture to the animal. The thing that you can do is determine whether it is a correlation (but then you take correlation, it may be true, or it may not be true); or you take a list, a rank order; and it has been a great worry to everybody to find a means of validating tissue culture in vitro results. It has been mentioned that a compound was sent to 12 laboratories and two got the same result and the others different results. That is one way to do it; however, even that is not really a very good one. How to validate is a very big problem. This type of thing, the way we usually do it, because everything we are doing in cultures, we tried to verify this "*in vivo*". Now, with morphological things, it is easier to verify. For example, if you get certain necroses in culture of certain neurons, you may get animals and you may look whether in the brain you may find similar things after certain situations occur or not. And if you do not find them, you say "fine". That may be happening in the tissue culture, but probably not in vivo. But if you want to be very precise, and get precise figures, it is very difficult. As a matter of fact, this particular necrosis that you were talking about is a fairly recent thing that we have found. But that is exactly what we are going to be doing. We will try to see if we can find similar types of morphological.

Dr. Bide: Have you looked at the effects of pesticides on other enzymes in the nervous tissue in addition to cholinesterase?

Dr. Fedoroff: We have not done that: we are just ready to do that; we are going to be looking at that. We have one toxicology graduate student who is going to start his graduate work this fall. This is part of his problem. I alluded to subacute testing and necrosis of nervous tissue not because of the phenomenon itself, but rather from the point

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of view that so often you may find many other things going along with the primary effects which you never expected. In toxicity testing, not everything is black and white.

Dr. T.W. Sawyer: When does one use *in vitro* techniques to replace animals in research?

Dr. Fedoroff: This is a very big problem. I think that, as Dr. Plaa said, and it is very correct, if you have a very specific question to answer, I think that *in vitro* techniques are excellent because you can answer precisely a probably very specific question. However, when your question is not so specific or is affecting systemic organization of the animal, then it becomes very difficult to answer with *in vitro* methods.

Question: How long would you allow animals to survive on an infectious disease study?

Dr. Rowsell: As a former microbiologist, from the time I began in diagnostic microbiology, we had to inject many materials for diagnosis into animals. One of the primary examples, of course, was tuberculosis, for which we used guinea pigs. There were many organisms that were injected into animals because that was the only way to find and to isolate them. However, since the inception of the embryonated egg, and since the advent of tissue culture, animal use for diagnosis has practically disappeared. I would say that in our microbiology departments across the country only a few animals are used in research exercises. That is one area where replacement techniques have had important input. As far as the study of the infectious disease process is concerned, the journal Laboratory Investigation often contains information on some of the infectious diseases where a tissue culture system showed specific

changes in specific cells having been used in the process.

If you are looking at the entire animal in an infectious disease process, when the end point comes depends on the clinical signs in the animal itself. There are some organisms that are extremely pathogenic and which produce a morbid animal very, very quickly. I think any morbid animal should be destroyed because there are changes going on in that animal that are really unrelated to the defence mechanism; everything is broken down and you just get a defused septicemia or bacteremia. One thing does bother me significantly, and that is that in many of our veterinary journals we still find that the end point is the death of the animal; and the authors indicate that these animals showed significant distress before they were allowed to die. I think it wrong ethically for the veterinary profession to allow that. I think the investigators must seek different end points.

Dr. Ron Lenniger: What is your definition of psychological well-being?

Dr. Rowsell: A definition of psychological well-being is difficult because, in many instances, we have yet to learn the behavioural needs of specific species. There are certain terms that are creeping into the description of the well-being of animals such as "unnatural behaviour", due to the fact that the animals are being kept in "unnatural conditions". The interpretation is that those animals kept in these unnatural conditions showing this unnatural behaviour may not be suffering. However, one can certainly observe abnormal behaviour such as stereotypic pacing, bar biting, etc.. While they may indeed be in distress, there are also reports in the literature that indicate that those individuals showing abnormal behaviour have high levels of circulating endorphins that may be reducing their anxiety significantly. The study of the stress-induced analgesia is a very hot subject at this

time. We have much to learn before we address the matter of psychological well-being. However, I have tried to demonstrate some examples of animal behaviour, in my presentation. For example it has been known for years that rats like to stand on their hind legs and when given the opportunity will do so but have been denied that activity in our present caging. Also, it has been shown that the gentle handling of animals can change the results of some pharmacological activities, and some physiological activities. The one that I remember best was the rabbit with atherosclerosis: the animal that was gentled on a daily basis and handled carefully, developed less atherosclerosis than did the rabbit that was left in the cage and just fed the atherogenic diet.

In the United States, a recent requirement for assurance of the psychological well-being of non-human primates, which has been included as an amendment to the Animal Welfare Act, is causing all kinds of problems. (They are attempting, as well, to address the issue of exercise for dogs). Although it has been a year since the amendment was passed, psychological well-being of primates still is not being addressed. At one meeting I attended they noted that 55 different species of primates are being used and all 55 have some peculiarities as far as behavioural requirements are concerned. This issue is a real "can of worms" and we are going to have to address it, not only from the animal welfare point of view, but also from its effect upon the research being conducted. I believe the scientific output and the scientific validity of the studies are extremely important; we are using the animals to get meaningful results.

I think we can look very critically at the need for social contact. One of the problems that bothers me in psychological studies and behavioural studies, is the isolation of the animal. The very severe effect that cage isolation has on the effect of various drugs has been well

documented in the literature. Enrichment of the environment of a rat, for example, by providing something through which it can crawl, providing nesting material and something to play with, is a measure we can take at this time with very little additional cost.

Dr. Lenniger: Do you know when the new amendment will be addressed in the U.S.?

Dr. Rowsell: It is my understanding that they have not yet addressed the issue of psychological well-being of non-human primates. The other aspects of the amendments are out for comment, with submission to be in, I think, by August 27. It is my understanding that they are going to consider these before they even address the subject of non-human primates.

Dr. Lenniger: What about the evaluation of irritants?

Dr. Plaa: (Evaluation) of an unknown irritant first of all depends on what the dose level is and there certainly should be a level for that compound in which it is not irritant. In a chronic toxicity study, you do not want to have overt toxicity early in your study; you want the subject to be able to survive -- most say greater than 90 days, some say one year, without showing this. Therefore, your doses will be selected in terms of when that irritation is starting to manifest itself.

Question: Irritating to skin?

Dr. Plaa: No irritant to mucous membranes. In that situation, usually with irritants of the nature that you have described, you are not going into chronic toxicity studies. How is the individual going to end up by being exposed to this material chronically. I would say that you

have the irritancy in going to limit or -- the amount that an individual could be exposed to. Consequently, the chronic toxicity that you are talking about should be at doses that are below those that produce the irritant. I can't envisage, if you have a highly irritating substance, that you are going to expose the animal to this material for 90 days or a year at doses that are irritating. The toxicity has already been observed. I think you would have to select doses that are below ... What you are interested in knowing is, if you find a threshold, at that level in which irritancy does not occur, does this also have toxic problems?

Dr. Ilse: Some of the anti-cancer agents, such as nitrogen mustard, and some of the other anti-cancer agents like milian are quite irritating. Nitrogen mustard is quite irritating; however, it is used all the time. It is used acutely for patients that are quite sick. It is also used for patients who are undergoing therapy. Therefore, it must have been tested for shall we say, sub-chronic toxicity. Their strategy would be to use doses that fall below their first effects, because you want to separate that effect from the chronic effect, so your doses might be far lower than those that provoke irritancy. After all, you are looking for a chronic effect, not an acute effect and the whole aim of chronic toxicity, what it is based on, is that your patient might be exposed to this drug for a long time. So when your patient is at an acute level you usually use very high levels of drugs. When you go to the next level, sub-chronic, the doses are lower but the time in question, or period of testing, is longer; and if you go to chronic, again, the doses are much, much lower. For some drugs that are tested we use the drug sub-chronically, several times below the suggested human dose.

Dr. Plaa: Irritants of the type that you are describing constitute a real problem in terms of toxicological evaluation and this is part of

the problem of what we have seen with the low tolerance, for instance. However, I would say that one of the things that is necessary is to establish first, does tolerance develop to the irritant properties of your compound? If an animal is repetitively exposed to this material, does he become tolerant to it? An example of that is with nitrous oxide, in which you can have the individual become tolerant to the irritant properties of the material. If you take a naive volunteer (this is in humans) and put him in that environment and he gasps and has all sorts of reactions. If a tolerance develops, and I go back to your chronic studies, then this is probably what you will have to end up doing. You take account of the tolerance and you increase the dosage schedule as the tolerance become evident.

Dr. Lilli: What recourse does an investigator have if there is a disagreement on a particular experiment with the local ACC?

Dr. Rowsell: I assume that this question is addressed to me, because the Canadian Council on Animal Care has been responsible for the development of our Guides plus the Ethics of Animal Experimentation monograph. Our Ethics document is under constant review, the last revision in September, 1986. The CCAC also has an appeal mechanism under which, if an Animal Care Committee refuses to approve a particular project, the investigator, through the ACC, can submit it to the Council. Such projects are in part reviewed by Council, and in some cases are sent to experts and a decision reached. Animal Care Committees can also seek Council's advice.

I know very well the project that you are discussing and the fact that CCAC's Ethics document says that unanesthetized animals should not be struck or beaten and that trauma investigations have to be controlled. I also know very well that there is a need for such studies in that we

have many thousands, if not millions, of animals out there each year that are being subjected to these processes with these various traps, irrespective of whether or not we agree with the final product (and I think that is a personal decision). I have always followed the philosophy that the fur looks better on an animal than for any purpose to which man puts it. However, we do know these traps are being used, they are part of a national picture and that the federal government feels it is necessary to support this (humane trap) research.

The procedure that you followed as far as your Animal Care Committee is concerned, is correct, and one which I hope other ACCs would emulate if they had such a dilemma: send the project to the Council with the notation that they (the ACC) could not in the present context approve this project. The CCAC will then have it reviewed. In the case of this one, the project was reviewed by the entire Council and outside experts and certain modifications were suggested. If our findings support that the project should go on, we send that information to the institution. If the institution still says "We still don't like it. We are not going to do it. We think there are problems with it from an ethical standpoint and from a procedural standpoint", they need not have that project pursued at their institution. The final decision is the authority of the institutional animal care committee, and no decision of Council will change that. However, we will assist anyone in trying to find solutions to these very thorny issues.

Dr. Hamilton: Must we (the ACC) provide an alternative to animal use and if so, where are we going to find it?

Dr. Rowsell: No. You (the ACC) do not have to provide an alternative. If you find a study distasteful and even, after review by our Council and the opinion of experts, you still do not want to approve that

study, the ACC can say "It will not be done". I can give you examples where projects have not been carried out because, while CCAC felt there could be support for the study, the local Animal Care Committee still demanded modifications by the investigator. It is up to the investigator to satisfy the ACC. Their responsibilities as an institutional animal care committee are being met as they interpret them, based on CCAC guidelines.

We would hope that you would try and find other ways and means of reaching the goals of the study. However, if you could not, then it is within your right to deny that study should be done in your institution.

Dr. Hamilton: What responsibility does a local ACC have regarding, for example, field use of animals or off-site contracts using animals?

Dr. Rowsell: Regarding the authority of the Animal Care Committee, our guidelines require that if projects are to be carried out in field locations or other laboratories, the institutional Animal Care Committee and the home institution should continue to have control over those projects. Unfortunately, we have had some investigators that have taken projects elsewhere and done them.

Another thing that concerns us is that some of our critics say that CCAC Animal Care Committee system does not work because there are not enough projects rejected. Our reply is that our system is working because, of the many projects that are discussed, a good many are modified and improvements are made. We do not publicize the number of cases that have been sent back for review, or the number of investigators that have been called in to answer the Committee's question. Rejection is a sign of failure on the part of the Animal Care Committee.

Question: What authority does an ACC have legally?

Dr. Rowsell: I don't know what your legal rights are. However, as far as our Council is concerned, that is within the requirements of our Guidelines: over the right of management. We have never been challenged in that particular area. Unfortunately, we have not got into a government policy decision where that research had to be done. We just about had that situation with a case of contract research from the Department of National Defence at the University of Ottawa on the dog radiation study. Again, through the "open door" policy concern and discussions with representatives of the animal welfare movement, the investigators and DND representatives, a modification was made that made that project acceptable.

Question: What are the requirements for long term toxicity testing?

Dr. Ilse: If you have a compound that is going to be administered on anything other than a one shot basis, even for short durations, say five or six days, at intervals, FDA will ask you for a two year mouse study.

Dr. Lorne Rowbottom: Who decides when and whether an *in vitro* test is an acceptable alternative to live animals use?

Dr. Plaa: I think the primary point of the validation is very critical and, as Dr. Ilse has said, WHO is to replace a procedure that is now being employed. It will take time in order to develop the confidence to accept that method as a valid method. However, if you talk in terms of relatively valid to do something limited, I think that is where we already see this. We see that in the short term, *in vitro* mutagenic tests. Even though we haven't validated them in

any sense precisely, we are satisfied that there is a certain degree of acceptability; therefore, we are using data that are coming from it; however, they are not replacing anything. What is going to come up with the Draize test and those in vitro techniques which are supposedly going to measure irritancy at a certain point is that, if they are to replace the rabbit eye as an irritancy test, then they will have to be compared and validated. And the ironic thing is, if the motive is to get rid of the irritancy test because of the use of the animals, to validate the other procedures you have to use more animals with compounds you already know. It is paradoxical, in that it takes an increased use of animals to put in something new.

Question: Again, though, when do you adopt new techniques: When you're forced to or when the new compound is just too toxic?

Dr. Plaa: You could look at it that way; it could be a compound. On the other hand, I am in the industry and I am convinced that if they end up by using this test instead of that, if they accept that and something happens, e.g. litigation, they are not going to care if it was valid. In 1987, they are expecting you to be using the techniques that are going to come out in 1995. People have been sued in the 1970s for not having used the techniques that only exist now. In a litigation situation, I think the question is, if you permit an invalidated method to be used instead of something else that they have been using at the time, you would have a tough time to defend yourself.

Dr. Ilse: The question was, well what are you doing with humans when you have tremendous diversity and life styles, and where endocrine function is affected by social mores? How do you treat this? If it is only through a wealth of experience and comparing the results that you get in all these models and then using that as a guide to very careful

clinical studies, what that really means is all of the testing takes a very long time. I don't see anyone in his right mind saying that in vitro tests can replace safety tests for a drug which has been used in a population ... It probably will get different results in a different population group.

Question: Who is held responsible for idiosyncratic drug reactions or very low probability side effects?

Dr. Rowsell: In that regard, there is one slide that I did not show today, which I think is very appropriate, concerning vaccine production. This came from the National Research Council in the United States: "The law today makes manufacturers liable for injuries caused by a vaccine, even if they were not negligent in designing it. The legal system has run amok and companies can't take the financial risk. So the law also has to change in its interpretation".

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DRES ANIMAL CARE COMMITTEE  
WORKSHOP ON ALTERNATIVES TO ANIMALS  
IN RESEARCH:  
ROUND TABLE RECOMMENDATIONS

UNCLASSIFIED

DRES ANIMAL CARE COMMITTEE WORKSHOP  
ON ALTERNATIVE TO ANIMALS IN RESEARCH:  
ROUND TABLE RECOMMENDATIONS

The DRES Animal Care Committee, invited speakers and several guests met in a round table discussion on the day following the workshop presentations. The purpose of this meeting was to provide recommendations to Chief/DRES and the DRES ACC concerning future directions in the use and care of research animals. The discussions were wide ranging and encompassed all areas of animal use and the types of experiments likely to be undertaken at DRES. The following specific recommendations were proposed by this *ad hoc* working group:

- a. a focus officer should be appointed to keep abreast of and report to the DRES ACC on developments in short term (toxicity) testing using *in vitro* or non-*in vivo* techniques;
- b. the DRES ACC should institute reciprocal arrangements with other institutions (e.g., ADRI, University of Calgary) for evaluation of animal use protocol applications. The working group felt that the small size of the research community at DRES could contribute to "genetic drift" as far as acceptable methods and experimental technique is concerned. The incorporation of advice from other (disinterested) institutions would help prevent this insular outlook;
- c. the use of *in vitro* or alternative methods to answer specific (rather than global) questions is both time and cost efficient. Management should actively encourage and require *in vitro* methods when feasible;

- d. Mechanistic studies (e.g., regulation of membrane integrity) are likely to all go *in vitro* and therefore, the adoption and acquisition of appropriate technology should be encouraged at DRES;
- e. the DRES ACC should open lines of communication with other defence communities to effect the timely exchange of information and ideas which may prevent needless duplication of effort and animal use. This may be accomplished through focus officers within existing international collaborative relationships; and
- f. animal care and welfare requirements in research environments are constantly changing and evolving. DRES management should appoint a focus officer (e.g., the vivarium supervisor) to responsible for reporting on new developments in hardware and the associated costs of acquisition and/or implementation before these developments become mandatory.

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This special publication presents the texts of papers presented to the Workshop on Alternatives to Animals held at Defence Research Establishment Suffield, Ralston, Alberta, on September 16-17, 1987. The papers presented by eminent Canadian scientists are concerned with the merits of alternatives to animals used in research. It also includes discussions after each presentation and recommendations emanating from the workshop.

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